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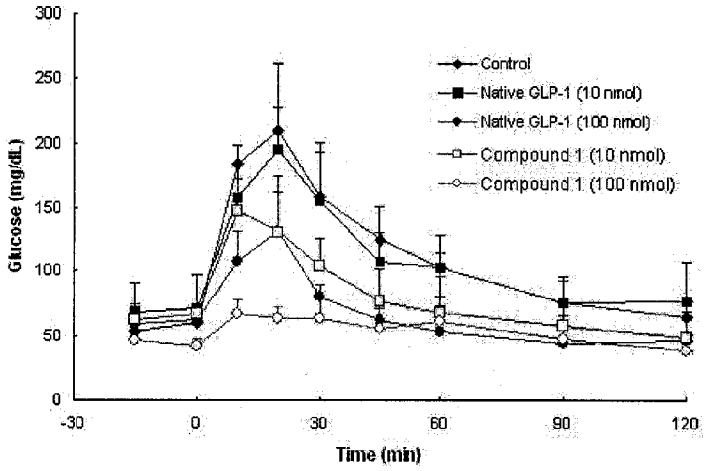
(54) Title: BIOACTIVE SUBSTANCE CARRIER FOR IN VIVO STABLE DELIVERY TEHREOF, CONJUGATE CONTAINING THE SAME, AND METHOD OF IN VIVO STABLE DELIVERY OF THE BIOACTIVE SUBSTANCE



(57) Abstract: The present invention provides a method of increasing in vivo half-life and stability of drugs by forming in vivo conjugation between disulfanyl group of modified bioactive substances and serum protein, especially free thiol group (Cys34) of serum albumin, i.e., 'new stable disulfide (S-S) covalent bond'. The invention provides efficient in vitro analysis methods capable of showing that the designed disulfanyl group is conjugated with serum protein in qualitative and quantitative ways. The invention provides a method of suitably designing bioactive substances on molecular level so that the bioactive substances and blood proteins can be efficiently in vivo conjugated via a disulfide covalent bond. The invention provides specific pharmacological treatment effects and treatment methods using the modified bioactive substances.

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TITLE OF THE INVENTION

BIOACTIVE SUBSTANCE CARRIER FOR *IN VIVO* STABLE DELIVERY TEHREOF, CONJUGATE CONTAINING THE SAME, AND METHOD OF *IN VIVO* STABLE DELIVERY OF THE BIOACTIVE SUBSTANCE

CROSS REFERENCE TO RELATED APPLICATION

This application claims priority to and the benefit of Provisional Patent Application No. 60/731,592 filed on October 27, 2005, which is hereby incorporated by reference for all purposes as if fully set forth herein.

BACKGROUND OF THE INVENTION

(a) Field of the Invention

This invention relates to a technology of modifying low-molecular-weight bioactive substances with short *in vivo* half-life and low stability in order to achieve a stable and efficient *in vivo* delivery thereof. More specifically, the present invention relates to a bioactive substance carrier capable of conjugating bioactive substances with a functional group on blood proteins, thereby conferring stability and desirable pharmacokinetic properties to the bioactive substances; a bioactive substance-bioactive substance carrier conjugate containing the bioactive substance carrier, and a low-molecular-weight bioactive substance which is available as a drug for treatment and prevention in mammals including human and selected from the group consisting of a natural substance, synthetic organic compound, nature-derived peptide and synthetic peptide; and a method for a stable and efficient *in vivo* delivery of the low-molecular-weight bioactive substance based on the use of the bioactive substance-bioactive substance carrier conjugate.

(b) Description of the Related Art

With the introduction of insulin, biopharmaceutics opened and has rapidly developed along with the advancement of life science and completion of human genome project and since 2000, biopharmaceutics more than 500 has been on clinical studies and approximately 10 treatments are approved by Food and Drug Administration (FDA) every

year. In particular, among the biopharmaceutics, peptide-based medicines are characterized by their strong treatment and preventive effects and biocompatibility, thereby being studied as new treatments or alternative treatments in the field of treatment and prevention for numerous disease symptoms.

However, as such peptide drugs and unstable low-molecular-weight drugs are easily biodegraded by various enzymes such as proteases present *in vivo*, they usually have short half-life. Furthermore, in the case of the peptide-class drugs, it is especially difficult to maintain their efficient concentration in blood in comparison with other low molecular drugs. Also, because they are macromolecules, their penetration into biological membranes is not easy, they may cause immunogenicity, and they generally have low solubility and thus their formulation has numerous restrictions. In particular, of the above mentioned drawbacks, the short half-life, low *in vivo* stability, and low bioavailability (BA) are recognized as portions to be improved in the development of prevention and treatment agents.

In general, most medicines provide their medicinal components into body in oral or injection form and they can exert treatment and prevention effects only when they are present within blood above a certain concentration. In many cases, high dose is given in order to increase the effect of treatment but it frequently involves various side effects and thus its use has the limits.

There have been proposed administration methods based on various drug delivery systems (DDS) such as slow-release capsule, depot and pump to improve the above-mentioned problems. However, such approaches have too many drawbacks to be generally applied so as to maintain the concentration of treatment medicines for a long-time period. For example, in case of skin adhesion dosage forms, they have to possess properties capable of penetrating medicines into skin tissues at a suitable rate when adhered to skin, and in slow-release dosage forms, dosage particles have time constraint for their release and are rapidly eliminated by macrophages when run into blood. If treatment medicines are to be administered by injection, many cases where prescription for injection is required repeatedly and continuously would not be advisable. In particular, self administration causes side effects due to its unskilled handling and in many cases, exhaustive personal training about administration methods is needed.

The development of drug delivery system which can efficiently improve the

drawbacks about the drugs themselves, i.e., short half-life, low *in vivo* stability and low bioavailability (BA) and repetitive injection administration method over long time period is desperately required.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a bioactive substance carrier capable of conjugating a low-molecular-weight bioactive substance useful in the body with a functional group on blood proteins, thereby conferring stability and desirable pharmacokinetic properties to the bioactive substance.

It is another object of the invention to provide a bioactive substance-bioactive substance carrier conjugate with improved *in vivo* stability and half-life, comprising the bioactive substance carrier.

It is still another object of the invention to provide a method for *in vivo* delivery of a bioactive substance comprising the steps of conjugating the bioactive substance with a specific functional group on blood proteins using the bioactive substance-bioactive substance carrier conjugate, to increase *in vivo* stability and half-life of the bioactive substance.

It is still another object of the invention to provide a method for simple and efficient *in vitro* analysis comprising the step of conducting *in vitro* analysis of a stable bond between the specific functional group of blood proteins and bioactive substance conjugate in quantitative and qualitative ways.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph showing the test results of blood glucose reducing effects according to each dose of Compound 1 of the present invention and native GLP-1 through oral glucose tolerance test (OGTT).

- control: administered with saline;
- each sample: intraperitoneally administered with native GLP-1 or Compound 1 at 15 min. before the test start;
- glucose is orally administered to all groups once at 0 min.; and
- the number of mice in each group: 4.

Fig. 2 is a graph showing long-acting degree of blood glucose reducing effects of

Compound 1 of the invention and native GLP-1.

- control: administered with saline;
- each sample: intraperitoneally administered with native GLP-1 or Compound 1 once at 15 min. before the test start;
- glucose is orally administered to all groups at 0 and 180 min., respectively; and
- the number of mice in each group: 4.

Fig. 3 is a graph showing long-acting degree of blood glucose reducing effects, comparing Compound 1 of the invention with native GLP-1 and d-ala-GLP-1 through intraperitoneal glucose tolerance test (IPGTT).

- control: administered with phosphate buffer saline;
- each sample: subcutaneously administered with native GLP-1, d-ala-GLP-1 or Compound 1 at 4 hours before the test start;
- glucose is intraperitoneally administered to all groups at 0 min.; and
- the number of mice in each group: 6-8.

Fig. 4 is a graph showing long-acting degree of blood glucose reducing effects, comparing Compound 7 and Compound 8 of the invention with Exendin-4 through intraperitoneal glucose tolerance test (IPGTT).

- control: administered with phosphate buffer saline;
- each sample: subcutaneously administered with Exendin-4, Compound 7 and Compound 8, respectively, at 9 hours before the test start;
- glucose is intraperitoneally administered to all groups at 0 min.; and
- the number of mice in each group: 8.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

A more complete appreciation of the invention, and many of the attendant advantages thereof, will be readily apparent as the same becomes better understood by reference to the following detailed description.

This invention relates to a technology of modifying low-molecular-weight bioactive substances with short *in vivo* half-life and low stability in order to achieve a stable and efficient *in vivo* delivery thereof. More specifically, the present invention relates to a bioactive substance carrier capable of conjugating bioactive substances with a

functional group on blood proteins, thereby conferring stability and desirable pharmacokinetic properties to the bioactive substances; a bioactive substance-bioactive substance carrier conjugate containing the bioactive substance carrier, and a low-molecular-weight bioactive substance which is available as a drug for treatment and prevention in mammals including human and selected from the group consisting of a natural substance, synthetic organic compound, nature-derived peptide and synthetic peptide; and a method for a stable and efficient *in vivo* delivery of the low-molecular-weight bioactive substance based on the use of the bioactive substance-bioactive substance carrier conjugate.

In the present invention, the bioactive substance carriers may bind to the bioactive substances *in vivo* through a linker. The bioactive substance carriers may comprise a reactive group capable of forming a stable covalent bond with a free group on blood component proteins that can form covalent bonds, thereby forming a stable covalent bond with the blood component proteins when administered into the body (blood), increasing *in vivo* half-life of the bioactive substances, and enhancing the stability thereof. In a preferred embodiment of the present invention, the bioactive substance carriers may form a stable covalent disulfide (S-S) bond with free thiol groups (Cys^{34}) of a serum protein, serum albumin, thereby remarkably enhancing *in vivo* stability of the bioactive substances bound thereto, and easily determining the presence of binding between the bioactive substances and the serum protein.

In general, low-molecular-weight bioactive substances having molecular weights of 100,000 or less have poor pharmacokinetic properties, such as, short *in vivo* half-life and instability, that is, they are readily degraded when administered in the body. For this reason, there have been difficulties in designing the low-molecular-weight bioactive substances as a drug, in spite of their excellent *in vivo* pharmacological effects. However, when such low-molecular-weight bioactive substances are conjugated with the bioactive substance carriers of the present invention, the bioactive substances can be stably bound to specific functional groups on blood proteins, whereby *in vivo* stability and half-life thereof can be remarkably increased.

More particularly, the present invention provides a bioactive substance carrier comprising a linker group capable of binding to low-molecular-weight bioactive substances; and a reactive group capable of forming a covalent bond with a functional

group on blood component proteins which is selected from the group consisting of hydroxyl group (-OH), thiol group (-SH), amino group (-NH_2) and carboxyl group (-CO_2H). In another aspect, the present invention provides a bioactive substance-bioactive substance carrier conjugate comprising the above-identified bioactive substance carrier; and a low-molecular-weight bioactive substance linked to the bioactive substance carrier through a linker group.

In another aspect, the invention provides *in vivo* delivery method of bioactive substances comprising the steps of forming a bioactive substance-bioactive substance carrier conjugate by binding the bioactive substance carrier of the present invention and a low-molecular-weight bioactive substance, to increase the half-life of the low-molecular-weight bioactive substance and improve the stability thereof; and administering the bioactive substance-bioactive substance carrier conjugate, and a composition for *in vivo* delivery of bioactive substances containing the bioactive substance-bioactive substance carrier conjugate. In the *in vivo* delivery method of bioactive substances and the composition for *in vivo* delivery of bioactive substances of the present invention, upon administration, the bioactive substances can form the stable covalent bond with the blood component protein, whereby the *in vivo* stability thereof the bioactive substances can be increased.

In still another aspect, the present invention provides a composition for treatment or prevention of diseases against which the bioactive substance has treatment activity, containing the bioactive substance-bioactive substance carrier conjugate, and a method for treatment or prevention of diseases against which the bioactive substance has treatment activity, by administering the bioactive substance-bioactive substance carrier conjugate.

Hereinafter, the invention will be described in more detail.

1. Definition of Terms

1) Bioactive Substances: As used herein, the term "bioactive substances" refers to all nature-derived or synthetic organic compounds and nature-derived or synthetic peptides having improvement, treatment and prevention effects on symptoms or diseases in mammals, especially, human, and in particular, to low molecular substances having molecular weights of 100,000 or less. More particularly, the bioactive substances of the

invention may be nature-derived natural substances, peptides, hormones, synthetic peptides, synthetic hormones and raw medicinal materials. For example, there may be included insulinotropic peptides such as glucagons like peptide-1 (GLP-1), glucagon family peptide hormones such as exendin-3 or exendin-4, which have blood glucose reducing effects in mammals, and LHRH (Luteinizing Hormone-Releasing Hormone). The administration of the bioactive substances together with the bioactive substance carriers or the bioactive substance-bioactive substance carrier conjugates according to the invention enables the bioactive substances to exert their inherent *in vivo* activity in more efficient way.

Glucagon like peptide-1 (GLP-1): GLP-1 is a intestinal hormone peptide that consists of 31 amino acids, and is released from proglucagon produced in L-cells of GI-tract. It decreases the blood glucose level by stimulating insulin depending on the concentration of glucose in blood, delays empty feeling in stomach, decreases intake of foods, and in particular, stimulates the functions of β-cells. Accordingly, the administration of the bioactive substance-bioactive substance carrier conjugates wherein GLP-1 is bound as a bioactive substance or GLP-1 together with the bioactive substance carriers can result in excellent blood glucose reducing effects, whereby high blood glucose-related diseases such as diabetes or obesity can be effectively treated or prevented.

Exendin-3 and Exendin-4 peptide: Exendin-3, Exendin-4 and derivatives thereof are poison components of *Heloderma suspectum* and they are nature-derived peptides consisting of 39 amino acids with blood glucose reducing effects. Accordingly, the administration of the bioactive substance-bioactive substance carrier conjugates wherein Exendin-3, Exendin-4 or derivatives thereof is bound as a bioactive substance or Exendin-3, Exendin-4 or derivatives thereof together with the bioactive substance carriers can result in excellent blood glucose reducing effects, whereby high blood glucose-related diseases such as diabetes can thus be effectively treated or prevented.

LHRH (Luteinizing Hormone-Releasing Hormone): LHRH is a hormone generated in hypothalamus stimulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior lobe of the pituitary. The agents of acetate thereof are effectively used to diagnose whether hypothalamus, pituitary gland and genital organs have abnormal or inactive functions and recently, they are used as treatments for disease symptoms such as prostate cancer, endometriosis and uterus myoma. Accordingly, the administration of the bioactive substance-bioactive substance carrier

conjugates wherein LHRH is bound as a bioactive substance or LHRH together with the bioactive substance carriers can play roles to treat or diagnose sex hormone-related diseases and control the ovulation period in mammals, and further, the diseases such as prostate cancer, endometriosis and uterus myoma can be effectively treated or prevented thereby.

2) Bioactive substance carrier: As used herein, the "bioactive substance carrier" or "bioactive substance delivery system" comprises a linker group and reactive group and increases *in vivo* stability and half-life of the bioactive substances to be conjugated therewith by linking them to specific functional groups on blood proteins. The definitions of the reactive group and the linker group in the invention follow.

3) Reactive group: As used herein, the term "reactive group" means all the chemical groups capable of forming a new and stable covalent bond with specific functional groups on blood component proteins present in the body, for example, hydroxyl group (-OH), thiol group (-SH), amino group ($-NH_2$) or carboxyl group ($-CO_2H$), preferably "S-S covalent bond" with free thiol group (-SH) on plasma proteins. In preferred embodiments, the reactive group is usually used to encompass all the chemical groups capable of forming a new "S-S covalent bond" by binding to plasma proteins, preferably, a free thiol group present on the 34^{th} cysteine residue of albumin protein in blood. The reactive groups of the invention are generally stable in aqueous solutions and are capable of forming a new "S-S covalent bond" by reacting with the free thiol groups (-SH group) on the plasma proteins. Also, the reactive group of the invention may include a linker group binding site, an S-S bond-forming site and, optionally, a leaving group that is separated after the formation of the stable S-S bond.

The reactive group may be selected from the group consisting of disulfanyl groups, and the disulfanyl groups may include 2-pyridyl disulfanyl group, *N*-alkylpyridinium disulfanyl group, 5-nitro-2-pyridyl disulfanyl group, 3-nitro-thiophenyl disulfanyl, 1-piperido disulfanyl group, 3-cyano-propyl disulfanyl group, 2-thiouredyl disulfanyl group, 4-carboxylbenzyl disulfanyl group, 1-phenyl-1*H*-tetrazolyl disulfanyl group, 1-amino-2-naphthyl disulfanyl group, 3-carboxyl-6-pyridyl disulfanyl group, 2-benzothiazolyl disulfanyl group, 4-nitro-thiophenyl disulfanyl group, and the like. The reactive group

may optionally contain a leaving group that is separated after the formation of the stable S-S bond by reaction with the free thiol groups on the plasma proteins.

4) Linker group: As used herein, the term "linker group" means all the chemical moieties that can be linked or bound to both of the reactive group of the bioactive substance delivery system and the bioactive substances. The linker group may include alkyl groups of C1 to C6 consisting of one or more methyl, ethyl, propyl, butyl, etc., alkoxy group, cycloalkyl group, polycyclic group, aryl group, polyaryl group, substituted aryl group, heterocyclic group and substituted heterocyclic group. In addition, the linker group may include poly ethoxy amino acids including (2-amino)ethoxy acetic acid (AEA). The preferable linker group of the invention may be $AE(E)_nA$ ([2-(2-amino)-ethoxy](ethoxy)$_n$ acetic acid) (n=0~2) containing one to three ethoxy groups. In particular, the use of AEEE acetic acid (AEEEA) increases of solubility, resulting in advantageous effects on the formation of stable covalent bonds between the bioactive substances and blood components, thereby exhibiting better blood glucose reducing effects than AEEA.

The linker group can link the bioactive substances and the reactive group by being bound to the terminals of the substances or positioning the inside of the substance.

5) Modified bioactive substances or Bioactive substance-bioactive substance carrier conjugate: As used herein, the term "modified bioactive substance" means all the bioactive substances modified by the attachment of preferred reactive group so as to be able to conjugate with functional groups of plasma proteins, preferably, thiol group (-SH). The modified bioactive substances may be in the form where the bioactive substances are linked to the reactive group via the linker group of the bioactive substance carrier (bioactive substance-bioactive substance carrier conjugate) or they are directly linked to the reactive group without the linker group. Those modified bioactive substances are designed so as to be stable against peptidases and such stability can be obtained by their ability to easily form the conjugation with free thiol groups on plasma proteins.

The modified bioactive substances to be mainly used in the present invention can include natural substances, synthetic low molecular compounds, nature-derived peptides, synthetic peptides and the like having molecular weights of 100,000 or less with

pharmacological activity that can be used with certain treatment or prevention purpose in mammals, preferably human. They may usually be used in the form of bioactive substance-bioactive substance carrier conjugates wherein the bioactive substance is linked to the reactive group via the linker group, or bound directly to the reactive group without the linker group. Also, in the present invention, the bioactive substances is modified such that the formation of selective 'S-S covalent bond' of plasma proteins can be directly or indirectly analyzed by simple *in vitro* qualitative and quantitative methods when treated *in vivo*.

6) Blood components: In the present invention, the bioactive substances have to form a stable bond with a specific functional group on proteins in blood (blood components) so that they can not be degraded *in vivo*, thereby being stabilized, and their half-life can increase. The blood components may be present in the mobile or fixed form in blood. The fixed blood components may include tissues membrane receptors, interstitial proteins, fibrin proteins, collagens, platelets, endothelial cells and epithelial cells that have no mobility in blood. Further, they may also include cell membranes, membrane receptors, somatic body cells, skeletal, smooth muscle cells, neuronal components, osteocytes and osteoclasts that are associated with the above examples. The mobile blood components are blood components capable of continuously locomoting without being fixed. In general, they are not associated with cell membranes and are present in the concentration of at least 0.1ug/ml in blood. The blood protein components, which can be used as mobile blood components in the invention, may include serum albumin, transferrin, ferritin, celuroplasmin and immunoglobulin such as IgM and IgG. Generally, *in vivo* half-life of the mobile blood components is at least 12 hours. Among them, the serum albumin is most preferable and it is most preferable to form a stable disulfide covalent bond with a free thiol group on cysteine present in the 34^{th} amino acid of the serum albumin.

7) Plasma protein: The plasma proteins mean all the proteins that are contained in plasma. Most plasma proteins present in blood are serum albumin and globulin. Approximately 7 g is contained within 100 uM of plasma. Albumin is contained in 50 to 70 %, globulin is in approximately 20 to 50 %, and fibrinogen is within 10 %. The blood

protein that does not contain the fibrinogen is called as 'serum protein'.

8) Functionality: In the present invention, the term "functionality" can be defined as functional groups on blood components, preferably plasma proteins, which can form a new and stable 'S-S covalent bond' through the reaction with reactive groups of modified bioactive substances. In general, various functional groups, such as, hydroxyl group (-OH), thiol group (-SH), amino group ($-NH_2$), carboxyl group ($-CO_2H$), and the like, exist on the plasma proteins. However, in the present invention, the reactive groups of the modified bioactive substances may usually react with a free thiol group (-SH) on plasma proteins, to form a new and stable 'S-S covalent bond'.

9) Protective group: In the present invention, the term "protective group" can be defined as a chemical functional group derived from the reaction among amino acids in the synthesis of peptides and its representative examples may include acetyl (Ac), fluorenylmethyloxy-acrbonyl (Fmoc), *t*-butyloxycarbonyl (Boc), benzyloxycarbonyl (Cbz), *t*-butyl (*t*-Bu), tri-phenylmethyl (Trt), 2,2,4,6,7-pentamethyldihydrobenzofuran-*S*-sulfonyl (Pbf), and the like. The general protective groups and abbreviations of the amino acids used in the present invention are summarized in Table 1 below.

Table 1. Natural amino acids and their abbreviations

Natural amino acids and their abbreviations			
Name	3-Letter abbreviation	1-Letter abbreviation	Protected amino acids
Alanine	Ala	A	Fmoc-Ala-OH
Arginine	Arg	R	Fmoc-Arg(Pbf)-OH
Asparagine	Asn	N	Fmoc-Asn(Trt)-OH
Aspartic acid	Asp	D	Asp(tBu)-OH
Cysteine	Cys	C	Fmoc-Cys(Trt)-OH
Glutamic acid	Glu	E	Fmoc-Glu(tBu)-OH
Glutamine	Gln	Q	Fmoc-Gln(Trt)-OH
Glycine	Gly	G	Fmoc-Gly-OH
Histidine	His	H	Fmoc-His(Trt)-OH
Isoleucine	Ile	I	Fmoc-Ile-OH
Leucine	Leu	L	Fmoc-Leu-OH
Lysine	Lys	K	Fmoc-Lys(Boc)-OH
Methionine	Met	M	Fmoc-Met-OH
Phenylalanine	Phe	F	Fmoc-Phe-OH
Proline	Pro	P	Fmoc-Pro-OH
Serine	Ser	S	Fmoc-Ser(tBu)-OH
Threonine	Thr	T	Fmoc-Thr(tBu)-OH
Trytophan	Trp	W	Fmoc-Trp(Boc)-OH
Tyrosine	Tyr	Y	Fmoc-Tyr(tBu)-OH
Valine	Val	V	Fmoc-Val-OH

2. Structural Form and Construction of Modified Bioactive Substances

The structural form and constitution of the modified bioactive substances of the invention (bioactive substance-bioactive substance carrier conjugates) can be illustrated as follows:

<Modified bioactive substances>

The "**X_1, bioactive substances**" are reaction compounds with molecular weights of 100,000 or less which exhibit physiological activity, and may refer to nature-derived natural substances, peptides, hormones, synthetic peptides, synthetic hormones and raw medicinal substances, which have pharmacological effects and can be efficiently used in the treatment and/or prevention against diseases in mammals, preferably human. For example, the bioactive substances may include peptides, such as glucagons like peptide-1

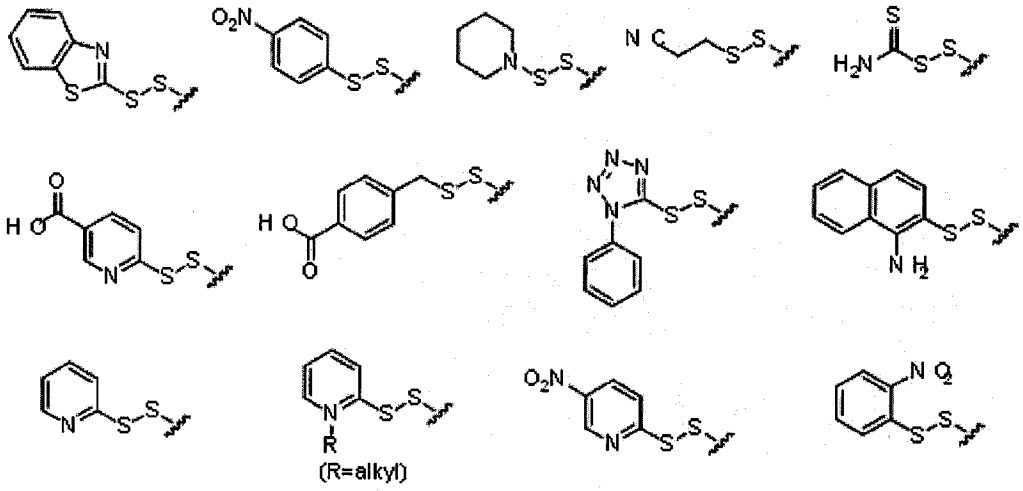
(GLP-1) or exendin-3 or -4, having blood glucose reducing effects in mammals.

The "**X_2, linker group**" refers to a linker group of positioning between the bioactive substances and the reactive group and connecting them through a chemical bond. The linker group may include alkyl group of C1 to C6 consisting of one or more methyl, ethyl, propyl, butyl, etc., alkoxy group, cycloalkyl group, polycyclic group, aryl group, polyaryl group, substituted aryl group, heterocyclic group, substituted heterocyclic group, and the like. In addition, the linker group may include poly ethoxy amino acids, such as (2-amino) ethoxy acetic acid (AEA). The preferred linker group of the invention may be $AE(E)_nA$ ([2-(2-amino)-ethoxy](ethoxy)$_n$ acetic acid) (n=0~2) containing one to three ethoxy groups. In particular, the use of AEEE acetic acid (AEEEA) increases solubility and has advantageous effects on the formation of stable covalent bonds between the bioactive substances and blood components, and thus, better blood glucose reducing effects can be obtained than AEEA.

The "**X_3, reactive group**" refers to all the chemical groups capable of forming a new and stable covalent bond with specific functional groups on blood component proteins in the body, such as, hydroxyl group (-OH), thiol group (-SH), amino group ($-NH_2$) or carboxyl group ($-CO_2H$), preferably "S-S covalent bond" with free thiol group (-SH group) on plasma proteins, as described above. In preferred embodiments, the reactive group may be a disulfanyl group which is generally stable in aqueous solutions and capable of forming a new "S-S covalent bond" by reacting with a free thiol group (-SH group) on blood proteins, preferably albumin.

For example, the reactive groups may include 2-pyridyl disulfanyl group, *N*-alkylpyridinium disulfanyl group, 5-nitro-2-pyridyl disulfanyl group, 3-nitro-thiophenyl disulfanyl, 1-piperido disulfanyl group, 3-cyano-propyl disulfanyl group, 2-thiouredyl disulfanyl group, 4-carboxyl-benzyl disulfanyl group, 1-phenyl-1*H*-tetrazolyl disulfanyl group, 1-amino-2-naphthyl disulfanyl group, 3-carboxyl-6-pyridyl disulfanyl group, 2-benzothiazolyl disulfanyl group, 4-nitro-thiophenyl disulfanyl group, and the like. The chemical structure of the above-mentioned reactive groups may be shown in the following formula 1, and they may optionally comprise a leaving group that is separated after the reaction with a free thiol group.

Formula 1: Illustration of Chemical Structure of Reactive Group (X_3)



3. Synthesis of Bioactive Substances

(1) Common Nomenclature of Peptides and Protective Group thereof

Unless specifically defined by the prefix 'D' (e.g., D-Ala or NMe-D-Ile), the stereochemistry of their α-carbon of all the amino acids and aminoacyl residues presented in the peptides described herein is natural or 'L' configuration.

The nomenclature and abbreviation of naturally existing or non-natural amino acids and the amino acids actually employed in the amino acid synthesis, used in the present invention, are represented in Table 1 above. D-type amino acids can be represented by three-English letter abbreviation or one-letter abbreviation (in corresponding small letter) using the prefix 'D'. For example, D-type alanine can be abbreviated as "D-Ala" or "a".

In the case that no abbreviation is represented in the above, nomenclature and abbreviation can be found in the document [Calbiochem-Novabiochem Corp. 1999 Catalog and Peptide Synthesis Handbook or the Chem-Impex International, Inc. Tools for Peptide amp; Solid Phase Synthesis 1998-1999 Catalogue].

(2) General Synthesis of Peptides

The consecutive condensation of amino acids performed in the invention may be carried out by automatic peptide synthesizer widely known in the relevant field or the applicant's automatic peptide synthesizer (Peptron, Inc., Korea; see Korean Patent

Application No. 2000-0049344, which is incorporated as a reference). Preferred synthesis conditions are as follows: treating α-amino acid protected by Fmoc group with a secondary amine solution, preferably piperidine to deprotect it, washing it with solvent in a sufficiently excessive amount, and performing coupling reaction in DMF solvent by adding about 5 times moles of coupling reagents and another respectively protected amino acid. The coupling reagents mainly used in the present invention may include *N, N`*-dicyclohexylcarbodiimide (DCC), *N,N`*-diisopropylcarbodiimide (DIC), *O*-benzotri-azol-1-yl-*N,N,N`,N`*-tetramethyluroniumhexafluorophosphate (HBTU), benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (PyBOP), benzo-triazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP), [*O*-(7-azabenzotri-azol-1-yl)-*N,N,N`,N`*-tetramethyluroniumhexafluorophosphate] (HATU), 1*H*-hydroxy-benzotriazole (HOBt), 1*H*-hydroxy-7-azabenzotriazole (HOAt), and the like, and if necessary, the reaction can be performed by addition of organic bases such as TEA, DIEA and NMM.

In the last step of peptide synthesis using solid phase resins in the present invention, the desired peptides may be removed from the resins by consecutive or one-time manipulation on polypeptides, and the peptides may be deprotected from the protective groups that protect each amino acid residue. For the removal of the polypeptides from the resins and deprotection of the peptides from the protective groups present in the residues, cleavage reagent cocktails that cut the bond of resins-polypeptides, for example, dichloromethane mixture cocktail solution containing trifluoroacetic acid (TFA), triisopropylsilane (TIS), thioanisole, water or ethanedithiol (EDT) may be used. The obtained mixture solution may produce precipitate by the treatment of excessive amount of diethylether solvent that has been refrigerated. The obtained precipitate may be centrifuged so that it is completely precipitated, and the excessive amounts of TFA, TIS, thioanisole, water, EDT and the like may be primarily removed and these procedures may be repeated two times or so, to obtain solid precipitate.

Completely deprotected peptide salts may be isolated and purified using reverse phase high performance liquid chromatography (HPLC) by running mixture solvent comprising water and acetonitrile solvents. The isolated and purified peptide solutions may be completely condensed and dried by lyophilization, to produce solid peptides.

4. Quantitative Analysis of Albumin and Bioactive Substance-Bioactive Substance Carrier Conjugate

The invention may also provide a method for effective *in vitro* quantitative analysis with regard to the disulfide complex wherein albumin and modified bioactive substance are *in vivo* conjugated with each other through disulfide bond.

The conventional analysis used to determine whether albumin and bioactive substances are conjugated to create a conjugation complex had experimental limits and problems where albumin complex needs to be separately purified and analyzed using LC-MS and MALDI-TOF. However, in the present invention, the disulfide bond between the conjugation complex may be selectively reduced by treatment of DTT (dithiothreitol; Cleland's reagent), thereby easily measuring the amount of the bioactive substances that are separated and released from the complex. This analysis has significant meaning in experiment in that the bioactive substances in conjugation with albumin can be effectively measured *in vitro* through HPLC analysis.

The present invention is further explained in more detail with reference to the following examples. These examples, however, should not be interpreted as limiting the scope of the present invention in any manner.

EXAMPLE

Example 1:

Compound 1: D-Ala^{8}-GLP-1 (7-36)-Lys^{37}-(ε-AEEA-PDSP)-NH_2.4TFA:
His-D-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Lys-(ε-AEEA-PDSP)-NH_2.4TFA.
(SEQ ID NO: 1) (PDSP: propionic disufanyl pyridine, same as below)

100 μmol of rink amide methylbenzhydrylamine (MBHA) resin (0.6 mmol/g, Novabiochem Corporation) were measured and put into a reaction vessel. The resin was solvated with 5 ml of DMF and allowed to be sufficiently swollen for 5 min. 3 ml of 20% piperidine DMF solution was added to the swollen resin, which was then shaken, and the piperidine solution was removed therefrom. Then, 20% piperidine DMF solution was added again and reaction was kept for 10 min, thereby completely eliminating Fmoc

protective group that protected the resin, and then, washed five times or more with 10 ml of DMF solvent. In this step, it was determined using Kaiser Test whether the Fmoc protective group is deprotected [E. Kaiser *et al.*, *Anal. Biochem.* (**1970**) *34*, 595].

Fmoc-Lys(Aloc)-OH (500 μmol), HOBt (500 μmol), HBTU (500 μmol) and DIEA (1 mmol) were completely dissolved in 5 ml of DMF solvent, and then, added to the resin which is deprotected from Fmoc protective group. The reaction solution was shaken at room temperature for 2 hours or so and then, washed with 10 ml of DMF solvent five times or more. In this step, Kaiser Test was performed in the same way as above to determine whether the coupling of Fmoc-amino acids occurs.

Next, coupling was successively carried out in accordance with the following synthesis cycle: (1) washing with DMF solvent (10 ml) five times or more; (2) deprotecting using 20% piperidine DMF solution (3 ml) two times for 10 min; (3) washing with DMF solvent (10 ml) five times or more; (4) adding Fmoc-amino acid; (5) activating the amino acid and coupling for 2 hours by addition of coupling reagent; and (6) washing with DMF solvent (10 ml) five times or more.

Step 1: Coupling Step

Amino acids (5 equivalents or more) protected with Fmoc were added to the resin reaction vessel in the following order and coupled: (1) Fmoc-Lys(Aloc)-OH; (2) Fmoc-Arg(Pbf)-OH; (3) Fmoc-Gly-OH; (4) Fmoc-Lys(tBoc)-OH; (5) Fmoc-Val-OH; (6) FmocLeu-OH; (6) Fmoc-Trp-OH; (7) Fmoc-Ala-OH; (8) Fmoc-Ile-OH; (9) Fmoc-Phe-OH; (10) Fmoc-Glu(OtBu)-OH; (11) Fmoc-Lys(tBoc)-OH; (12)Fmoc-Ala-OH; (13) Fmoc-Ala-OH; (14) Fmoc-Gln(Trt)-OH; (15) Fmoc-Gly-OH; (16) Fmoc-Glu(OtBu)-OH; (17) Fmoc-Leu-OH; (18) Fmoc-Tyr(tBu)-OH; (19) Fmoc-Ser(tBu)-OH; (20) Fmoc-Ser(tBu)-OH; (21) Fmoc-Val-OH; (22) Fmoc-Asp(OtBu)-OH; (23) Fmoc-Ser(tBu)-OH; (24) Fmoc-Thr(tBu)-OH; (25)-Fmoc-Phe-OH; (26) Fmoc-Thr(tBu)-OH; (27) Fmoc-Gly-OH; (28) Fmoc-Glu(OtBu)-OH; (29) Fmoc-D-Ala-OH; (30) Boc-His(N-Trt)-OH.

Step 2: Selective Deprotection Step of Aloc Group

After 300 μmol of $Pd(PPh_3)_4$ was dissolved in 5 ml of CH_3Cl:NMM:AcOH (18:1:0.5), it was added to the resin synthesized in Step 1 and shaken at room temperature for 2 hours or more. The reaction resin was washed with $CHCl_3$ (10 ml, six times); 20%

acetic acid CH_2Cl_2 solution (10 ml, six times); CH_2Cl_2 (10 ml, six times); and DMF (10 ml, six times or more). The occurrence of the selective deprotection of Aloc group was determined by carrying out Kaiser Test in the same way as above.

Step 3: Introduction Step of Linker Group

After Fmoc-(AEEA)-OH (Fmoc-miniPEG-OH, 3 mmol), HOBt (3 mmol), HBTU (3 mmol) and DIEA (6 mmol) were completely dissolved in 10 ml of DMF solvent, and added to the resin deprotected from Fmoc protective group. The reaction solution was shaken at room temperature for 4 hours or more and then washed with 10 ml of DMF solvent ten times or more. In this step, Kaiser Test was performed in the same way as above to determine the occurrence of the coupling of Fmoc-amino acids. The reaction solution was treated with 10 ml of 20% piperidine DMF solution, shaken for 30 min or more thereby to remove Fmoc protective group, and then, washed with 10 ml of DMF five times or more.

N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP, 2 mmol) purchased from Pierce Biotechnology was dissolved in 5 ml of CH_2Cl_2 solvent, reacted with the resin synthesized as above for 3 hours or more with shaking, and then, and washed with CH_2Cl_2 (10 ml) six times or more.

Step 4: Cleavage Step

Immediately upon the completion of synthesis, the resin coupled with the peptides was cleaved by using the mixture of TFA/water (95:5) for 3 hours. The obtained mixture solution was treated with excessive amount of diethyl ether solvent that had been refrigerated, to generate a precipitate. The obtained precipitate was centrifuged so as to be completely precipitated, and the excessive amount of TFA was primarily eliminated, and these procedures were repeated two times or so, whereby solid peptides were obtained.

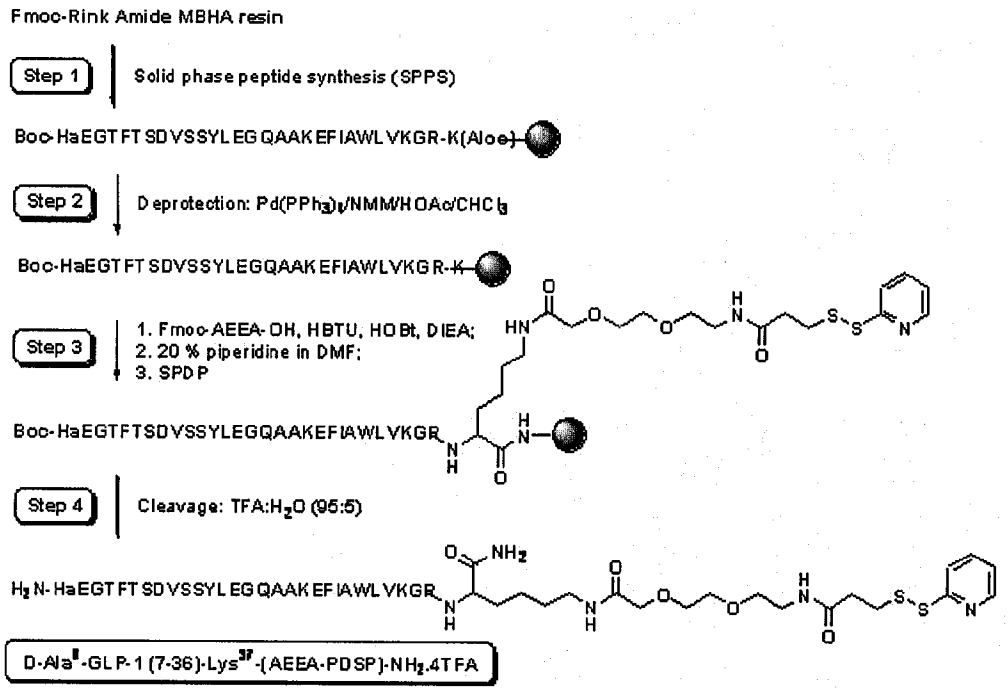
The obtained peptides were purified with HPLC using C-18 columns and an acetonitrile/water concentration gradient solvent system containing 0.01% TFA over 50 min, wherein the concentration gradient ranges from 5% to 100%. The purified pure fractions were lyophilized, to obtain Compound 1, D-Ala^8-GLP-1 (7-36)-Lys^{37}-(ε-AEEA-PDSP)-NH_2.4TFA in the form of white powder of TFA salts:

Compound 1: D-Ala^8-GLP-1 (7-36)-Lys^{37}-(ε-AEEA-PDSP)-NH_2.4TFA:

Rt=23.63 min (various concentration gradients ranging from 5% to 100% of acetonitrile/water containing 0.01% TFA over 30 min); MALDI-TOF= 3,769.

The synthesis of Compound 1 described in the above can be summarized as reaction formula 1 shown in below.

Reaction Formula 1. Synthesis of D-Ala^8-GLP-1 (7-36)-Lys^{37}-(AEEA-PDSP)-NH_2.4TFA (Compound 1)



The following peptides were prepared in accordance with the same synthesis procedures as above.

Compound 2: D-Ala^8-GLP-1 (7-36)-Lys^{37}-(ε-AEEEA-PDSP)-NH_2.4TFA: His-D-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Lys-(ε-AEEEA-PDSP)-NH_2.4TFA (SEQ ID NO: 2). Rt=23.51 min (various concentration gradients ranging from 5% to 100% of acetonitrile/water containing 0.01% TFA over 30 min); MALDI-TOF= 3,814.

Compound 3: D-Ala^{8}-Lys^{26}-(ε-AEEA-PDSP)-GLP-1 (7-36)-NH_2.4TFA: His-D-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-(ε-AEEEA-PDSP)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-NH_2.4TFA (SEQ ID NO: 3). Rt=23.78 min (various concentration gradients ranging from 5% to 100% of acetonitrile/water containing 0.01% TFA over 30 min); MALDI-TOF= 3,641.

Compound 4: D-Ala^{8}-Lys^{26}-(ε-AEEEA-PDSP)-GLP-1 (7-36)-NH_2.4TFA: His-D-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys(ε-AEEEA-PDSP)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-NH_2.4TFA (SEQ ID NO: 4). Rt=23.66 min (various concentration gradients ranging from 5% to 100% of acetonitrile/water containing 0.01% TFA over 30 min); MALDI-TOF= 3,686.

Compound 5: GLP-1 (7-36)-Lys^{37}-(ε-AEEA-PDSP)-NH_2.4TFA: His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Lys-(ε-AEEA-PDSP)-NH_2.4TFA (SEQ ID NO: 5). Rt=23.61 min (various concentration gradients ranging from 5% to 100% of acetonitrile/water containing 0.01% TFA over 30 min); MALDI-TOF= 3,769.

Compound 6: GLP-1 (7-36)-Lys^{37}-(ε-AEEEA-PDSP)-NH_2.4TFA: His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Lys-(ε-AEEEA-PDSP)-NH_2.4TFA (SEQ ID NO: 6). Rt=23.49 min (various concentration gradients ranging from 5% to 100% of acetonitrile/water containing 0.01% TFA over 30 min); MALDI-TOF= 3,814.

Compound 7: Exendin-4 (1-39)-Lys^{40}-(ε-AEEA-PDSP)-NH_2.5TFA: His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Lys-(ε-AEEA-PDSP)NH_2.5TFA (SEQ ID NO: 7). Rt=15.68 min (various concentration gradients ranging from 5% to 100% of acetonitrile/water containing 0.01% TFA over 30 min); MALDI-TOF =4,658.

Compound 8: Exendin-4 (1-39)-Lys^{40}-(ε-AEEEA-PDSP)-NH_2.5TFA:

His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Lys-(ε-AEEEA-PDSP)-NH_2.5TFA (SEQ ID NO: 8). Rt=17.19 min (various concentration gradients ranging from 5% to 100% of acetonitrile/water containing 0.01% TFA over 30 min); MALDI-TOF =4,702.

Compound 9: Lys^{27}-(ε-AEEA-PDSP)-Exendin-4 (1-39)-NH_2.5TFA: His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-(ε-AEEA-PDSP)-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2.5TFA (SEQ ID NO: 9). Rt=21.81 min (various concentration gradients ranging from 5% to 100% of acetonitrile/water containing 0.01% TFA over 30 min); MALDI-TOF =4,533.

Compound 10: Lys^{27}-(ε-AEEEA-PDSP)-Exendin-4 (1-39)-NH_2.5TFA: His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-(ε-AEEEA-PDSP)-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2.5TFA (SEQ ID NO: 10). Rt=21.75 min (various concentration gradients ranging from 5% to 100% of acetonitrile/water containing 0.01% TFA over 30 min); MALDI-TOF =4,577.

Compound 11: Exendin-3 (1-39)-Lys^{40}-(ε-AEEA-PDSP)-NH_2.5TFA: His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Lys-(ε-AEEA-PDSP)NH_2.5TFA (SEQ ID NO: 11). Rt=20.73 min (various concentration gradients ranging from 5% to 100% of acetonitrile/water containing 0.01% TFA over 30 min); MALDI-TOF =4,676.

Compound 12: Exendin-3 (1-39)-Lys^{40}-(ε-AEEEA-PDSP)-NH_2.5TFA: His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Lys-(ε-AEEEA-PDSP)-NH_2.5TFA (SEQ ID NO: 12). Rt=20.68 min (various concentration gradients ranging from 5% to 100% of acetonitrile/water containing 0.01%

TFA over 30 min); MALDI-TOF =4,721.

Compound 13: Lys^{27}-(ε -AEEA-PDSP)-Exendin-3 (1-39)-NH_2.5TFA: His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-(ε-AEEA-PDSP)-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2.5TFA (SEQ ID NO: 13). Rt=20.65 min (various concentration gradients ranging from 5% to 100% of acetonitrile/water containing 0.01% TFA over 30 min); MALDI-TOF =4,547.

Compound 14: Lys^{27}-(ε-AEEEA-PDSP)-Exendin-3 (1-39)-NH_2.5TFA: His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2.5TFA (SEQ ID NO: 14). Rt=20.55 min (various concentration gradients ranging from 5% to 100% of acetonitrile/water containing 0.01% TFA over 30 min); MALDI-TOF =4,592.

Example 2

Compound 15: Leuprolide-GSG-Lys-(ε-AEEA-PDSP)-NH_2.2TFA: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Gly-Ser-Gly-Lys(ε-AEEA-PDSP)-NH_2.2TFA (SEQ ID NO: 15). (Pyr=pyroglutamic acid, pE)

100 μmol of rink amide MBHA resin (0.6 mmol/g, Novabiochem Corporation) were measured and put into a reaction vessel. The resin was solvated with 5 ml of DMF and allowed to be sufficiently swollen for 5 min. 3 ml of 20% piperidine DMF solution was added to the swollen resin, which was then shaken and the piperidine solution was removed therefrom. Then, 20% piperidine DMF solution was added again and reaction was kept for 10 min, thereby completely eliminating Fmoc protective group that protected the resin and then, washed five times or more with 10 ml of DMF solvent.

Fmoc-Lys(Aloc)-OH (500 μmol), HOBt (500 μmol), HBTU (500 μmol) and DIEA (1 mmol) were completely dissolved in 5 ml of DMF solvent, and then, added to the resin which is deprotected from Fmoc protective group. The reaction solution was shaken at room temperature for 2 hours or so and then, washed with 10 ml of DMF solvent five times

or more. In this step, Kaiser Test was performed in the same way as above to determine whether the coupling of Fmoc-amino acids occurs.

Next, coupling was successively carried out in accordance with the following synthesis cycle: (1) washing with DMF solvent (10 ml) five times or more; (2) deprotecting using 20% piperidine DMF solution (3 ml) two times for 10 min; (3) washing with DMF solvent (10 ml) five times or more; (4) adding Fmoc-amino acid; (5) activating the amino acid and coupling for 2 hours by addition of coupling reagent; and (6) washing with DMF solvent (10 ml) five times or more.

Step 1: Coupling Step

Amino acids (5 equivalents or more) protected with Fmoc were added to the resin reaction vessel in the following order and coupled: (1) Fmoc-Lys(Aloc)-OH; (2) Fmoc-Gly-OH; (3) Fmoc-Ser(tBu)-OH; (4) Fmoc-Gly-OH; (5) Fmoc-Pro-OH; (6) Fmoc-Arg(Pbf)-OH; (6) Fmoc-Leu-OH; (7) Fmoc-D-Leu-OH; (8) Fmoc-Tyr(tBu)-OH; (9) Fmoc-Ser(tBu)-OH; (10) Fmoc-Trp(Boc)-OH; (11) Fmoc-His(Trt)-OH; (12) Boc-Pyr(tBu)-OH.

Step 2: Selective Deprotection Step of Aloc Group

After 300 μmol of $Pd(PPh_3)_4$ was dissolved in 5 ml of CH_3Cl:NMM:AcOH (18:1:0.5), it was added to the resin synthesized in Step 1 and shaken at room temperature for 2 hours or more. The reaction resin was washed with $CHCl_3$ (10 ml, six times); 20% acetic acid CH_2Cl_2 solution (10 ml, six times); CH_2Cl_2 (10 ml, six times); and DMF (10 ml, six times or more). The occurrence of the selective deprotection of Aloc group was determined by carrying out Kaiser Test in the same way as Example 1.

Step 3: Introduction Step of Linker Group

After Fmoc-(AEEA)-OH (Fmoc-miniPEG-OH, 3 mmol), HOBt (3 mmol), HBTU (3 mmol) and DIEA (6 mmol) were completely dissolved in 10 ml of DMF solvent, and added to the resin deprotected from Fmoc protective group. The reaction solution was shaken at room temperature for 4 hours or more and then washed with 10 ml of DMF solvent ten times or more. In this step, Kaiser Test was performed in the same way as above to determine the occurrence of the coupling of Fmoc-amino acids. The reaction solution was treated with 10 ml of 20% piperidine DMF solution, shaken for 30 min or

more thereby to remove Fmoc protective group, and then, washed with 10 ml of DMF five times or more.

N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP, 2 mmol) purchased from Pierce Biotechnology was dissolved in 5 ml of CH_2Cl_2 solvent, reacted with the resin synthesized as above for 3 hours or more with shaking, and then, and washed with CH_2Cl_2 (10 ml) six times or more.

Step 4: Cleavage Step

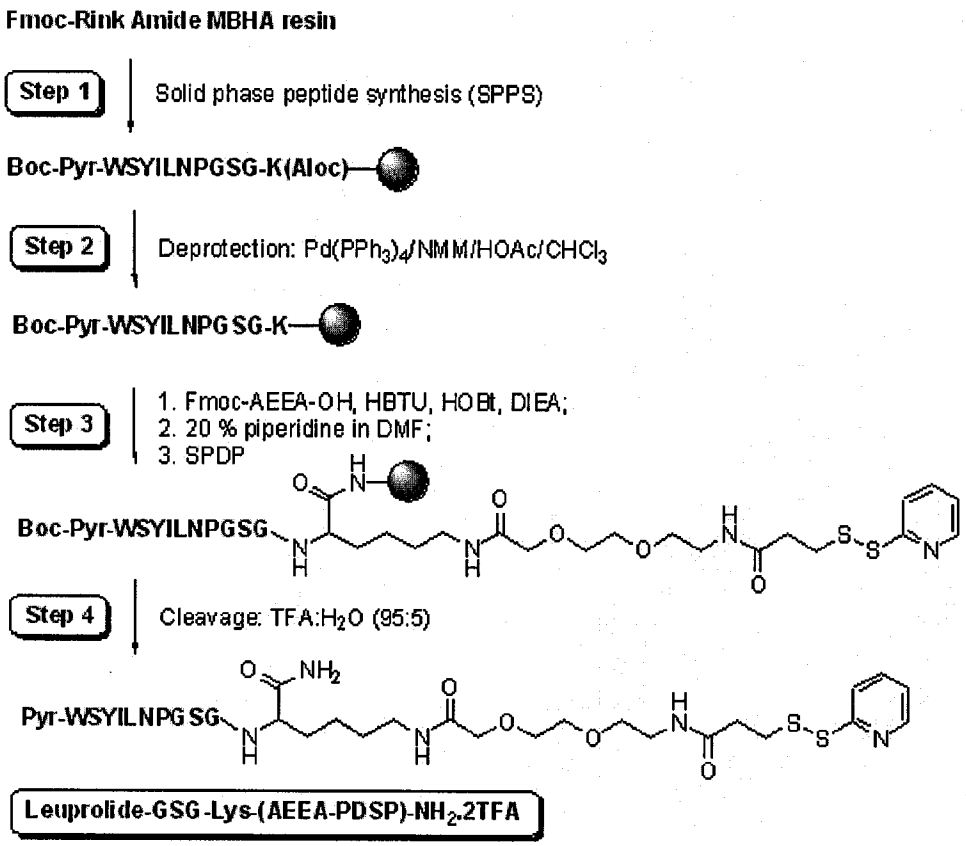
Immediately upon the completion of synthesis, the resin with the peptides coupled was cleaved using the mixture of TFA/water (95:5) for 3 hours. The obtained mixture solution treated with excessive amount of diethyl ether solvent that had been refrigerated to produce a precipitate. The obtained precipitate was centrifuged so as to be completely precipitated, the excessive amount of TFA was primarily eliminated, and these procedures were repeated two times or so, whereby solid peptides were obtained.

The obtained peptides were purified with HPLC using C-18 columns and an acetonitrile/water concentration gradient solvent system containing 0.01% TFA over 50 min, wherein the concentration gradient ranges from 5% to 100%. The purified pure fractions were lyophilized, to obtain bioactive substance Compound 15, Leuprolide-GSG-Lys-(ε-AEEA-PDSP)-NH_2.2TFA in the form of white powder of TFA salts.

Compound 15: Leuprolide-GSG-Lys-(ε-AEEA-PDSP)-NH_2.2TFA: Rt=23.63 min (various concentration gradients ranging from 5% to 100% of acetonitrile/water containing 0.01% TFA over 30 min); MS(ESI)m/e, $[M+H]^+$= 1853.

The synthesis of Compound 15 described in the above can be summarized as reaction formula 2 shown in below.

Reaction Formula 2. Synthesis of Leuprolide-GSG-Lys-(ε-AEEA-PDSP)-NH_2.2TFA (Compound 15)



The following peptides were prepared in accordance with the same synthesis procedures as above.

Compound 16: Leuprolide-GSG-Lys-(ε-AEEEA-PDSP)-NH_2.2TFA: Pyr-His-Trp-Ser-Tyr-Leu-Leu-Arg-Pro-Gly-Ser-Gly-Lys(ε-AEEEA-PDSP)-NH_2.2TFA (SEQ ID NO: 16). (Pyr=pyroglutamic acid)

Compound 17: Leuprolide-GG-Lys-(ε-AEEA-PDSP)-NH_2.2TFA: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Gly-Gly-Lys(ε-AEEA-PDSP)-NH_2.2TFA (SEQ ID NO: 17). (Pyr=pyroglutamic acid)

Compound 18: Leuprolide-GG-Lys-(ε-AEEEA-PDSP)-NH_2.2TFA:

Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Gly-Gly-Lys(ε-AEEEA-PDSP)-NH_2.2TFA (SEQ ID NO: 18). (Pyr=pyroglutamic acid)

Compound 19: Leuprolide-Lys-(ε-AEEA-PDSP)-NH_2.2TFA:

Pyr-His-Trp-Ser-Tyr-Leu-Leu-Arg-Pro-Lys(ε-AEEA-PDSP)-NH_2.2TFA (SEQ ID NO: 19). (Pyr=pyroglutamic acid)

Compound 20: Leuprolide-Lys-(ε-AEEEA-PDSP)-NH_2.2TFA:

Pyr-His-Trp-Ser-Tyr-Leu-Leu-Arg-Pro-Lys(ε-AEEEA-PDSP)-NH_2.2TFA (SEQ ID NO: 20). (Pyr=pyroglutamic acid)

Example 3

3.1. Albumin Binding Test:

It could be determined by measuring the degree of albumin binding that *in vivo* stability of Compound 15 (Leuprolide-GSG-Lys-(ε-AEEA-PDSP)-NH_2.2TFA), a modified bioactive substance synthesized in Example 2 above of the present invention, is increased by conjugation with a free thiol group (Cys^{34}) on human serum albumin (HSA). Further, this experiment could show that the stability of Compound 15 synthesized according to the present invention has increased by more effectively binding to albumin than unmodified Leuprolide. In addition, the quantity and presence of binding could be determined *in vitro* through simple HPLC analysis.

The conventional analysis used to determine the presence of the conjugation complex of albumin-bioactive substances have had experimental limits and problems and for example, albumin complex needs to be separately purified and analyzed using LC-MS and MALDI-TOF. However, the albumin-binding test according to the present invention is experimentally meaningful in that the presence of conjugation can be easily determined *in vitro* through simple pre-treatment of sample and HPLC analysis.

(1) Preparation of Stock Solutions:

Human serum albumin (HSA, 1 mM) solution was prepared by dissolving 66.5 mg of HSA (Sigma Aldrich) in PBS buffer (pH 7.2, 1 ml). Stock solutions (1 mM) of the modified bioactive substance Compound 15 (1.8 mg/ml) and Leuprolide (1.2 mg/ml) were

respectively prepared by the same method.

(2) Albumin Binding Test:

The stock solutions prepared in the above method were diluted with PBS buffer so that they had the compositions shown in Table 2 below thereby to prepare sample solutions (100 μl) for determination. Then, each reaction mixture was mixed and slowly shaken in an incubator for 30 min while the temperature condition of 37 ℃ was kept. After incubation, methanol (150 μl) was added to each sample vial, which was then voltexed for 10 min, to precipitate HSA. The precipitated albumin was spun down by centrifugation (12,000 rpm, 10 ℃, 10 min) and the supernatants were taken and analyzed using HPLC under the same conditions.

Table 2. Composition of Stock Solutions

	Albumin	Compound 15	Leuprolide	Buffer (PBS)
Control	50 ul		10 1	40 ul
1	50 ul	5 ul	-	45 ul
2	50 ul	10 ul	-	40 ul
3	50 ul	20 ul	-	30 ul
4	50 ul	40 ul	-	10 ul
5	50 ul	50 ul	-	-
6	40 ul	50 ul	-	10 ul
7	10 ul	-	-	90 ul
8	-	10 ul		90 ul

(3) Results:

The degree of conjugation was examined with increasing the concentration of the bioactive substance compounds at the fixed concentration of albumin. As the result, it could be considered that the degree of disulfide conjugation closely relates to the conditions of albumin used in the test, and in particular, it is associated with the content of the bioactive substance with respect to the free thiol groups. When the concentration of the bioactive substances represented as Compound 15 is 10 nmole or below, all amount of Compound 15 was conjugated with albumin, when the concentration thereof is 20 nmole or higher, Compound 15 that was not conjugated with albumin becomes observed, and then, there was a positive correlation wherein Compound 15 that was not conjugated increased as the amount to be added increased. The above-mentioned results are summarized in Table 3 below. According to the test results, about 34% of the HSA used was reacted with the bioactive substance compounds, and it is seemed that the bioactive substance

compounds selectively react with free thiol group on the 34th position of albumin.

Table 3. Albumin Binding Test Results

Bioactive Substance Compound15/albumin[*1]	$PA^{20.6min}$[*2]	$PA^{20.6min}/4.4M$[*3]	
5/50	0	0	
10/50	0	0	
20/50	1.3M	0.3	
40/50	9.8M	2.2	
50/50	14.7M	3.3	
50/40	17.9M	4.0	
10/0	4.4M	1.0	Compound 15 only

[*1]Albumin: Sigma A1653, remainder mostly globulins fraction V power albumin;

[*2]PA: peak area; and

[*3]M: million.

3.2. Quantitative Analysis of Albumin-Bioactive Substance Conjugation Complex

The disulfide complex of albumin of albumin and modified bioactive substance Compound 15 (Leuprolide-GSG-Lys-(ε-AEEA-PDSP)-NH_2.2TFA) which was conjugated through the experiment of above Example 3.1 was treated with DTT (dithiothreitol; Cleland's reagent) to selectively reduced the bond presented in the disulfide complex, whereby the amount of bioactive substance Compound 15 that is separated and released from the disulfide complex could be easily quantified. This analysis is experimentally meaningful in that bioactive substances which are conjugated with albumin can be effectively qualified *in vitro* through a simple method, such as HPLC analysis.

(1) Preparation of Stock Solutions:

HSA (1 mM) solution was prepared by dissolving 66.5 mg HSA (Sigma Aldrich) in PBS buffer (pH 7.2, 1 ml). Stock solutions of 1 mM modified bioactive substance Compound 15 (1.8 mg/ml) and 100 mM DTT (15.4 mg/ml) were respectively prepared in accordance with the same method.

(2) Quantitative Measurement of Albumin-Bioactive Substance Compound 15 Conjugation Complex:

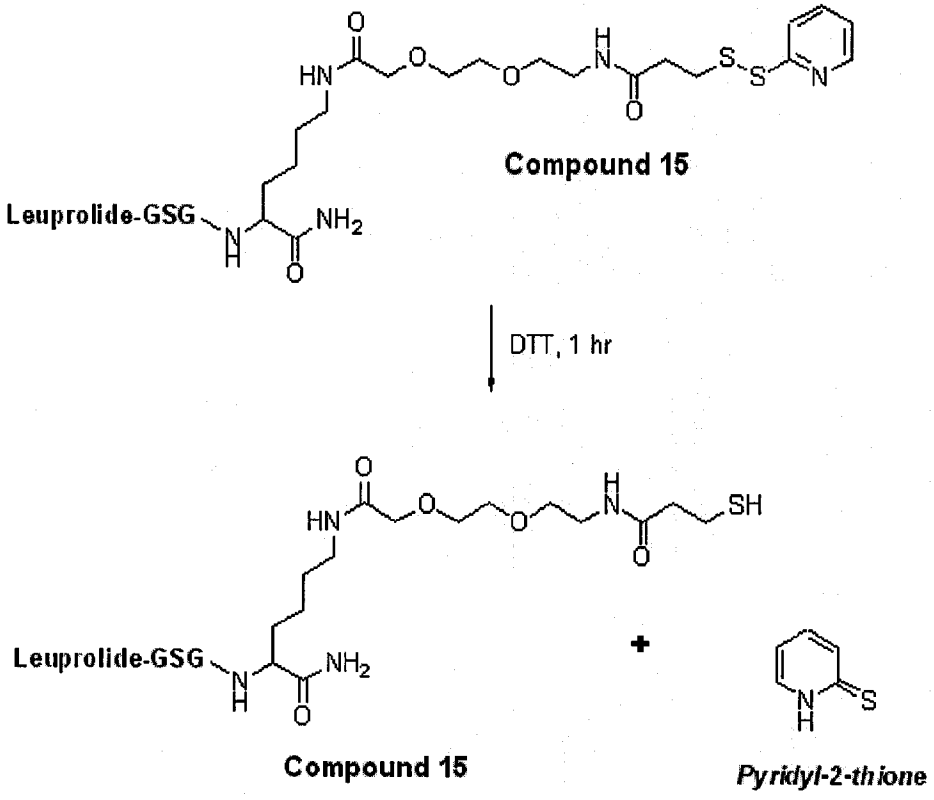
Albumin solution (50 μl) and bioactive substance Compound 15 solution (10 μl)

were mixed in each of 10 tubes, using the stock solutions prepared in the above method in the same conditions as used in the albumin binding test of Example 2, and then, incubated at 37 ℃ for 30 min while being slowly shaken.

After incubation, DTT was added thereto in amounts of 0 nmole, 100 nmole (2X), 200 nmole (4X), 500 nmole (10X), and 1,000 nmole, respectively, and reacted for about 1 hour at 37 ℃. After 1 hour elapsed, 25 μl was taken out from each sample, 50 μl of MeOH was added thereto, and the mixture was voltexed, to precipitate HSA. The precipitated albumin was spun down by centrifugation (12,000 rpm, 10 ℃, 10 min), and the supernatants were taken and analyzed using HPLC under the same conditions.

Quantitative analysis procedures including treatment of DTT used to determine the presence of disulfide conjugation between albumin and the bioactive substances in the present invention can be illustrated as Reaction Formula 3 below.

Reaction Formula 3. DTT Treatment Quantitative Analysis of Conjugation Complex of Albumin-Bioactive Substances



In the binding test of albumin and bioactive substance Compound 15, it was observed that 0.34 mmole of Compound 15 was bound to 1 mmole of albumin. In consideration of the characteristics of albumin and the test results, it is assumed that Compound 15 formed a disulfide conjugation by binding to Cys on the 34th position of albumin. If albumin and Compound 15 are conjugated by disulfide bond as above-mentioned, it can be inferred that Compound 15-1 having free thiol group could be released from disulfide conjugation by treatment of DTT reagent of a suitable concentration. With this aim, albumin and Compound 15 were incubated for 30 min to form disulfide conjugation. Then, after the addition of four different amounts of DTT ranging from 100 nmole to 1,000 nmole, they were incubated for 1 hour. When treating with DTT at a high concentration of 1,000 nmole or higher, albumin protein was degraded by DTT, and thus, albumin precipitation was formed in large amounts, whereby the analysis and observation were not easy. The test results are shown in Table 4 below. As summarized in Table 4, Compound 15-1 that was expected to be released at all the DTT treatment concentrations was observed through HPLC analysis, and Compound 15-1 expected to be released was readily observed when treated with high concentration, in comparison with when treated with low concentration.

Table 4. DTT Treatment Results

Compound 15[*1]/albumin[*2]	DTT(0.1 M)	Compound 15-1
10 μl/50 μl	0	Not detected
10 μl /50 μl	1 μl (2 X)	Detected
10 μl /50 μl	2 μl (4 X)	Detected
10 μl /50 μl	5 μl (10 X)	Detected
10 μl /50 μl	10 μl (20 X)	Detected

[*1,*2] 1mM in PBS [*2]Albumin: Sigma A1653, remainder mostly globulins fraction V power albumin

Example 4: Animal Test

4.1. Test for Measuring Blood Glucose Reducing Activity through OGTT:

4.1.1. Methods

Acute blood glucose reducing effects and the long-acting of the activity were

tested in this example, to determine the blood glucose reducing activity of Compound 1 of the present invention. As control samples for determining blood glucose reducing activity and the long-acting of the activity, native glucagon-like peptide-1 (GLP-1, 7-36) was used, and the activity of each peptide sample was measured by oral glucose tolerance test (OGTT).

ICR female mice (6 weeks old, Hanlim Experimental Animal Inc., Seoul, Korea) were employed as test animals, after being adapted in lab for 7 days and starved for 18 hours before the use of this test. Predetermined amount of each peptide sample was intraperitoneally administrated and, after 15 min, glucose (1.5 g/kg of mouse in 10 mM phosphate buffered saline, pH 7.4) was orally administrated (the time when glucose was administrated is defined as 0 min). At each determined time, blood was collected from caudal vein and the level of glucose therein was measured using glucometer (Accucheck Sensor, Roche). The experiments for the determination of blood glucose reducing activity and the long-acting of the activity are summarized in Table 5.

Table 5. Blood glucose Decrease Activity Measurement Sample

Group[1]	Sample	Dose (nmol/kg)	Glucose Administration Time (min)
1	Control[2]	-	0
2	Native GLP-1	10	0
3	Native GLP-1	100	0
4	Compound 1	10	0
5	Compound 1	100	0
6	Control[2]	-	0, 180
7	Native GLP-1	100	0, 180
8	Compound 1	100	0, 180

[1] n=4 in each group; and

[2] Control-saline administration group.

4.1.2. Results: Hypoglycemic effects

The activity of each peptide sample was measured through oral glucose tolerance test (OGTT) measurement method, and using native glucagon-like peptide-1 (GLP-1, 7-36) as a control sample, to determine the acute blood glucose reducing effects and the long-acting of the activity as tests, in order to determine the blood glucose activity of Compound 1 of the present invention.

First, animal test results with respect to the acute blood glucose reducing effects was measured through oral glucose tolerance test (OGTT), where each of Compound 1 of the invention and native GLP-1 was administered into mice in the amounts of 10 and 100 nmol/kg, respectively, and the results are shown in Fig. 1. In Fig. 1, control group was a group administrated with saline instead of Compound 1 or native GLP-1, and each group was intraperitoneally administrated with each test compound before 15 min and orally administrated with glucose one time at 0 min. The number of mice in each group was 4. The group administrated with native GLP-1 (10 nmol) showed no significant difference in blood glucose reducing curve from the control group administrated with saline only. The group administrated with native GLP-1 (100 nmol) showed significant difference from the control group and its blood glucose at the peak after 20 min of glucose administration was decreased by about 40% in comparison with the control group. On the contrary, the group administrated with 10 nmol of Compound 1 showed blood glucose reducing effects similar to the group administrated with native GLP-1 (100 nmol), and the group administrated with Compound 1 (100 nmol) showed no remarkable change, maintaining the blood glucose level of basal level before the administration of glucose for 120 min. Accordingly, Compound 1 has remarkably excellent blood glucose control effects in comparison with native GLP-1 based on the dose of 100 nmol.

Such result that Compound 1 has superior blood glucose control effects in comparison with native GLP-1 is considered to be caused by the fact that *in vivo* stability of Compound 1 is remarkably increased by *in vivo* conjugation of 2-pyridyl disulfanyl group of Compound 1 with free thiol group (Cys^{34}) of albumin present in blood through a new 'disulfide covalent bond,' when the compound was administrated in the same amount as native GLP-1, in comparison with the native GLP-1 that has very short half-life and thus poor *in vivo* stability.

Also, long-acting effects about blood glucose reducing activity of Compound 1 were shown in Fig. 2, compared with native GLP-1. In Fig. 2, each sample was administrated once before 15 min, glucose was administered at 0 min and 180 min, respectively, and the number of mice in each group was 4. While the group administrated with native GLP-1 showed blood glucose level similar to the control group when glucose was administrated at 180 min, the group administrated with Compound 1 showed significant difference from the control group in blood glucose reducing effects.

Accordingly, it could be verified that Compound 1 can maintain *in vivo* activity for longer time than native GLP-1. This result shows that while native GLP-1 is rapidly destructed or loses its functions *in vivo* due to its short half-life, Compound 1 has more extended half-life *in vivo* and thereby, continues blood glucose reducing effects. Such results are also considered to be caused by the fact that *in vivo* stability of Compound 1 is remarkably increased by *in vivo* conjugation of 2-pyridyl disulfanyl group of Compound 1 with free thiol group (Cys^{34}) of albumin present in blood via a new 'disulfide covalent bond'.

4.2. Test of Measuring Blood glucose Reducing Activity by IPGTT:

4.2.1. Methods

Long-acting of the blood glucose reducing effect was tested in this example to determine long-acting of the blood glucose reducing activity of Compounds 1, 7 and 8 of the present invention. As control samples for determining blood glucose reducing activity and long-acting of the activity, native GLP-1, d-ala-GLP-1, and Exendin-4 were used, and the activity of each peptide sample was measured by intraperitoneal glucose tolerance test (IPGTT).

ICR female mice (6 weeks old, Hanlim Experimental Animal Inc., Seoul, Korea) were employed as test animals, after being adapted in lab for 7 days. Before the test, 8 mice were picked out of each group and blood was collected from tail to measure the glucose concentration in blood using glucometer (Accucheck Sensor, Roche), and then, starved for 15 to 18 hours. Then, predetermined amount of each peptide sample was subcutaneously administrated and after 4 hours or 9 hours, glucose (2 g/kg of mouse in PBS, pH 7.2) was intraperitoneally administrated (the time when glucose was administrated is defined as 0 min). At each determined time, blood was corrected from caudal vein and the level of glucose therein was measured with glucometer. The experiments for the determination of blood glucose reducing activity and the long-acting of the activity are summarized in Table 6.

Table 6. Blood glucose Decrease Activity Measurement Sample

Group[1]	Sample	Dose (nmol/kg)	Glucose Administration Time (hr)
1	Control[2]	-	4hr, 9hr
2	Native GLP-1	100	4hr

3	d-ala-GLP-1	100	4hr
4	Compound 1	100	4hr
5	Exendin-4	10	9hr
6	Compound 7	10	9hr
7	Compound 8	10	9hr

[1] n=8 in each group, and

[2] Control-saline administration group

4.2.2. Results: Hypoglycemic effects

This example is to determine the blood glucose activity of Compounds 1, 7 and 8 of the present invention, wherein the activity of each peptide sample was measured through intraperitoneal glucose tolerance test (IPGTT) measurement method using Native GLP-1, d-ala-GLP-1, Exendin-4 as control samples to determine long-acting of blood glucose decrease activity.

The animal test results with respect to the long-acting of blood glucose reducing effect, when Compound 1 of the invention, native GLP-1 and d-ala-GLP-1 were respectively administered into mice in the amount of 100 nmol/kg, were measured through IPGTT, and shown in Fig. 3. In Fig. 3, the control group was a group administrated with saline instead of Compound 1, native GLP-1 or d-ala-GLP-1, each group was subcutaneously administrated with each test compound before 4 hours, and then, was intraperitoneally administrated with glucose once at 0 min. The number of mice in each group was 8. The groups administrated with native GLP-1 and d-ala-GLP-1 (100 nmol) showed no significant difference in blood glucose reducing curve from the control group administrated with saline only. In contrast, the group administrated with 100 nmol of Compound 1 showed significant reducing profile in blood glucose reducing curve even after 4 hours. Accordingly, Compound 1 has remarkably excellent blood glucose control effects in comparison with native GLP-1 and d-ala-GLP-1 based on the dose of 100 nmol.

Next, animal test results with respect to the long-acting of blood glucose reducing effects, when Compounds 7 and 8, and Exendin-4 were respectively administered into mice in the amounts of 10 nmol/kg, were shown in Fig. 4. In Fig. 4, the control group was a group administrated with saline instead of Compounds 7 and 8 or Exendin-4, each sample was subcutaneously administrated with each test compound before 9 hours, and then, intraperitoneally administrated with glucose once at 0 min. The number of mice in

each group was 8. The group administrated with Exendin-4 showed only a slight reducing effect in blood glucose reducing curve in comparison with the control group administrated with saline only, and there was no statistically significant difference therebetween. In contrast, the group administrated with 10 nmol of Compound 7 or 8 showed significant reducing profile in blood glucose reducing curve even after 9 hours, and especially, medicinal efficacies increased in proportion with the length of linkers. Accordingly, Compounds 7 and 8 have remarkably excellent blood glucose control effects in comparison with exendin-4 based on the dose of 10 nmol.

The result that Compounds 1, 7 and 8 have superior blood glucose control effects in comparison with native GLP-1 and Exendin-4 is considered to be caused by the fact that *in vivo* stability of Compounds 1, 7 and 8 is remarkably increased by *in vivo* conjugation of 2-pyridyl disulfanyl group of Compounds 1, 7 and 8 with free thiol group (Cys^{34}) of albumin present in blood via a new 'disulfide covalent bond,' when the compounds were administrated in the same amount as native GLP-1 and Exendin-4, in comparison with native GLP-1 and Exendin-4 that have very short half-life and thus poor *in vivo* stability.

WHAT IS CLAIMED IS:

1. A bioactive substance carrier comprising:

a linker group capable of binding to a low-molecular-weight bioactive substance having a molecular weight of 100,000 or less, which is selected from the group consisting of a natural or synthetic peptide, natural or synthetic hormone, and raw medicinal substance; and

a reactive group capable of forming a covalent bond with a functional group selected from the group consisting of hydroxyl group (-OH), thiol group (-SH), amino group ($-NH_2$) and carboxyl group ($-CO_2H$) on an blood component protein.

2. The bioactive substance carrier according to claim 1, wherein the blood component protein is serum albumin, transfferin, celuroplasmin or immunoglobulin, and the reactive group forms a disulfide covalent bond (S-S) with a free thiol group (-SH) on cysteine residue on the blood component protein.

3. The bioactive substance carrier according to claim 2, wherein the blood component protein is serum albumin, and the reactive group forms a stable disulfide covalent bond (S-S) with a free thiol group on the 34^{th} cysteine of the serum albumin.

4. The bioactive substance carrier according to claim 1, wherein the reactive group comprises:

a binding site to the linker group;

a covalent bond-forming site with the blood component protein; and

a leaving group that is separated after the formation of a stable covalent bond.

5. The bioactive substance carrier according to claim 1, wherein the reactive group is a disulfanyl group.

6. The bioactive substance carrier according to claim 5, wherein the disulfanyl group is selected from the group consisting of 2-pyridyl disulfanyl group, *N*-alkylpyridinium disulfanyl group, 5-nitro-2-pyridyl disulfanyl group, 3-nitro-thiophenyl

disulfanyl, 1-piperido disulfanyl group, 3-cyano-propyl disulfanyl group, 2-thiouredyl disulfanyl group, 4-carboxylbenzyl disulfanyl group, 1-phenyl-1*H*-tetrazolyl disulfanyl group, 1-amino-2-naphthyl disulfanyl group, 3-carboxyl-6-pyridyl disulfanyl group, 2-benzothiazolyl disulfanyl group and 4-nitro-thiophenyl disulfanyl group.

7. The bioactive substance carrier according to claim 1, wherein the linker group is selected from the group consisting of alkyl groups of C1 to C6, alkoxy groups, cycloalkyl groups, polycyclic groups, aryl groups, polyaryl groups, substituted aryl groups, heterocyclic groups, substituted heterocyclic groups, and ([2-(2-amino)-ethoxy] $(ethoxy)_n$ acetic acid) represented by $AE(E)_nA$ (n is 0, 1 or 2).

8. The bioactive substance carrier according to claim 7, wherein the linker group is AEEA or AEEEA.

9. A bioactive substance-bioactive substance carrier conjugate comprising:

the bioactive substance carrier according to any one of claims 1 to 8; and

a low-molecular-weight bioactive substance having a molecular weight of 100,000 or less selected from the group consisting of a natural or synthetic peptide, natural or synthetic hormone and raw medicinal substance and linked to the bioactive substance carrier via a linker group of the bioactive substance carrier.

10. The bioactive substance-bioactive substance carrier conjugate according to claim 9, wherein the bioactive substance is selected from the group consisting of insulinotropic peptide, glucagon family peptide hormone and LHRH (Luteinizing Hormone-Releasing Hormone).

11. The bioactive substance-bioactive substance carrier conjugate according to claim 10, wherein the bioactive substance is selected from the group consisting of glucagons like peptide-1 (GLP-1), exendin-3, exendin-4 and LHRH (Luteinizing Hormone-Releasing Hormone).

12. The bioactive substance-bioactive substance carrier conjugate according

to claim 10, which is selected from the group consisting of:

Compound 1: His-D-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Lys-(ε-AEEA-PDSP)-NH_2.4TFA;

Compound 2: His-D-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Lys-(ε-AEEEA-PDSP)-NH_2.4TFA;

Compound 3: His-D-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-(ε-AEEEA-PDSP)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-NH_2.4TFA;

Compound 4: His-D-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys(ε-AEEEA-PDSP)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-NH_2.4TFA;

Compound 5: His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Lys-(ε-AEEA-PDSP)-NH_2.4TFA;

Compound 6: His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Lys-(ε-AEEEA-PDSP)-NH_2.4TFA;

Compound 7: His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Lys-(ε-AEEA-PDSP)NH_2.5TFA;

Compound 8: His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Lys-(ε-AEEEA-PDSP)-NH_2.5TFA;

Compound 9: His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-(ε-AEEA-PDSP)-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2.5TFA;

Compound 10: His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-(ε-AEEEA-PDSP)-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2.5TFA;

Compound 11: His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-

Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Lys-(ε-AEEA-PDSP)NH_2.5TFA;

Compound 12: His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Lys-(ε-AEEEA-PDSP)-NH_2.5TFA;

Compound 13: His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-(ε-AEEA-PDSP)-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2.5TFA;

Compound 14: His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-(ε-AEEEA-PDSP)-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2.5TFA;

Compound 15: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Gly-Ser-Gly-Lys(ε-AEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid, pE);

Compound 16: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Gly-Ser-Gly-Lys(ε-AEEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid);

Compound 17: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Gly-Gly-Lys(ε-AEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid);

Compound 18: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Gly-Gly-Lys(ε-AEEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid);

Compound 19: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Lys(ε-AEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid); and

Compound 20: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Lys(ε-AEEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid).

13. A method for *in vivo* delivery of bioactive substance comprising the steps of:

forming a bioactive substance-bioactive substance carrier conjugate by binding the bioactive substance carrier according to claim 1 and a low-molecular-weight bioactive substance having a molecular weight of 100,000 or less, to increase the half-life of the low-molecular-weight bioactive substance and improve the stability thereof, and

administering the bioactive substance-bioactive substance carrier conjugate,

wherein the bioactive substance carrier comprises a linker group and a reactive

group,

the linker group is a functional group that binds to the bioactive substance, the reactive group comprises a binding site to the linker group, and a covalent bond-forming site capable of forming a stable covalent bond with a blood component protein, and

upon administration, the stable covalent bond with the blood component protein is formed, whereby *in vivo* stability of the bioactive substance is increased.

14. The method for *in vivo* delivery of bioactive substance of claim 13, further comprising the step of separating a leaving group from the reactive group of the bioactive substance carrier after the reactive group forms the stable covalent bond with the blood component protein.

15. The method for *in vivo* delivery of bioactive substance of claim 13, wherein the blood component protein is selected from the group consisting of serum albumin, transfferin, celuroplasmin and immunoglobulin, the covalent bond is a disulfide (S-S) covalent bond, and the reactive group forms a stable S-S covalent bond with a free thiol group (-SH) on cysteine residue of the blood component protein.

16. The method for *in vivo* delivery of bioactive substance of claim 15, wherein the blood component protein is serum albumin and the reactive group forms a stable S-S bond with a free thiol group on the 34^{th} cysteine of the serum albumin.

17. The method for *in vivo* delivery of bioactive substance of claim 13, wherein the reactive group is a disulfanyl group.

18. The method for *in vivo* delivery of bioactive substance of claim 17, wherein the disulfanyl group is selected from the group consisting of 2-pyridyl disulfanyl group, *N*-alkylpyridinium disulfanyl group, 5-nitro-2-pyridyl disulfanyl group, 3-nitro-thiophenyl disulfanyl, 1-piperido disulfanyl group, 3-cyano-propyl disulfanyl group, 2-thiouredyl disulfanyl group, 4-carboxylbenzyl disulfanyl group, 1-phenyl-1*H*-tetrazolyl disulfanyl group, 1-amino-2-naphthyl disulfanyl group, 3-carboxyl-6-pyridyl disulfanyl group, 2-benzothiazolyl disulfanyl group, and 4-nitro-thiophenyl disulfanyl group.

19. The method for *in vivo* delivery of bioactive substance of claim 13, wherein the linker group is selected from the group consisting of alkyl group of C1 to C6, alkoxy group, cycloalkyl group, polycyclic group, aryl group, polyaryl group, substituted aryl group, heterocyclic group, substituted heterocyclic group, and ([2-(2-amino)-ethoxy] $(ethoxy)_n$ acetic acid) represented by $AE(E)_nA$ (n is 0, 1 or 2)

20. The method for *in vivo* delivery of bioactive substance of claim 12, wherein the bioactive substance is selected from the group consisting of insulinotropic peptide, glucagon family peptide hormone, and LHRH (Luteinizing Hormone-Releasing Hormone).

21. The method for *in vivo* delivery of bioactive substance of claim 20, wherein the bioactive substance is selected from the group consisting of glucagons like peptide-1 (GLP-1), exendin-3, exendin-4 and LHRH (Luteinizing Hormone-Releasing Hormone).

22. A composition for delivery of bioactive substance comprising the bioactive substance-bioactive substance carrier conjugate according to claim 9, and forming a stable covalent bond with a blood component protein, whereby *in vivo* stability of the bioactive substance is increased.

23. The composition for delivery of bioactive substance of claim 22, wherein the bioactive substance is selected from the group consisting of insulinotropic peptide, glucagon family peptide hormone, and LHRH (Luteinizing Hormone-Releasing Hormone).

24. The composition for delivery of bioactive substance of claim 23, wherein the bioactive substance is selected from the group consisting of glucagons like peptide-1 (GLP-1), exendin-3, exendin-4, and LHRH (Luteinizing Hormone-Releasing Hormone).

25. The composition for delivery of bioactive substance of claim 22, wherein the bioactive substance-bioactive substance carrier conjugate is selected from the group

consisting of:

Compound 1: His-D-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Lys-(ε-AEEA-PDSP)-NH_2.4TFA;

Compound 2: His-D-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Lys-(ε-AEEEA-PDSP)-NH_2.4TFA;

Compound 3: His-D-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-(ε-AEEEA-PDSP)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-NH_2.4TFA;

Compound 4: His-D-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys(ε-AEEEA-PDSP)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-NH_2.4TFA;

Compound 5: His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Lys-(ε-AEEA-PDSP)-NH_2.4TFA;

Compound 6: His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Lys-(ε-AEEEA-PDSP)-NH_2.4TFA;

Compound 7: His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Lys-(ε-AEEA-PDSP)NH_2.5TFA;

Compound 8: His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Lys-(ε-AEEEA-PDSP)-NH_2.5TFA;

Compound 9: His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-(ε-AEEA-PDSP)-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2.5TFA;

Compound 10: His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-(ε-AEEEA-PDSP)-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2.5TFA;

Compound 11: His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-

Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Lys-(ε-AEEA-PDSP)NH_2.5TFA;

Compound 12: His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Lys-(ε-AEEEA-PDSP)-NH_2.5TFA;

Compound 13: His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-(ε-AEEA-PDSP)-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2.5TFA;

Compound 14: His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-(ε-AEEEA-PDSP)-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2.5TFA;

Compound 15: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Gly-Ser-Gly-Lys(ε-AEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid, pE);

Compound 16: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Gly-Ser-Gly-Lys(ε-AEEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid);

Compound 17: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Gly-Gly-Lys(ε-AEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid);

Compound 18: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Gly-Gly-Lys(ε-AEEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid);

Compound 19: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Lys(ε-AEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid); and

Compound 20: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Lys(ε-AEEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid).

26. A pharmaceutical composition for treatment of diabetes, reducing blood glucose or stimulating insulin secretion, comprising an effective amount of the bioactive substance-bioactive substance carrier conjugate according to claim 9 as an active ingredient, wherein the bioactive substance-bioactive substance carrier conjugate contains insulinotropic peptide as a bioactive substance.

27. The pharmaceutical composition according to claim 26, wherein the bioactive substance-bioactive substance carrier conjugate is selected from the group

consisting of:

Compound 1: His-D-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Lys-(ε-AEEA-PDSP)-NH_2.4TFA;

Compound 2: His-D-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Lys-(ε-AEEEA-PDSP)-NH_2.4TFA;

Compound 3: His-D-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-(ε-AEEEA-PDSP)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-NH_2.4TFA;

Compound 4: His-D-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys(ε-AEEEA-PDSP)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-NH_2.4TFA;

Compound 5: His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Lys-(ε-AEEA-PDSP)-NH_2.4TFA;

Compound 6: His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Lys-(ε-AEEEA-PDSP)-NH_2.4TFA;

Compound 7: His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Lys-(ε-AEEA-PDSP)NH_2.5TFA;

Compound 8: His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Lys-(ε-AEEEA-PDSP)-NH_2.5TFA;

Compound 9: His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-(ε-AEEA-PDSP)-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2.5TFA;

Compound 10: His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-(ε-AEEEA-PDSP)-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2.5TFA;

Compound 11: His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-

Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Lys-(ε-AEEA-PDSP)NH_2.5TFA;

Compound 12: His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Lys-(ε-AEEEA-PDSP)-NH_2.5TFA;

Compound 13: His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-(ε-AEEA-PDSP)-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2.5TFA; and

Compound 14: His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-(ε-AEEEA-PDSP)-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2.5TFA.

28. A pharmaceutical composition for diagnosis or treatment of sex hormone-related disease or control of ovulation period in mammals, comprising an efficient amount of the bioactive substance-bioactive substance carrier conjugate according to claim 9 as an active ingredient, wherein the bioactive substance-bioactive substance carrier conjugate contains LHRH (Luteinizing Hormone-Releasing Hormone) as a bioactive substance.

29. The pharmaceutical composition according to claim 28, wherein the sex hormone-related disease is prostate cancer, endometriosis, or uterus myoma.

30. The pharmaceutical composition according to claim 28, wherein the bioactive substance-bioactive substance carrier conjugate is selected from the group consisting of:

Compound 15: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Gly-Ser-Gly-Lys(ε-AEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid, pE);

Compound 16: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Gly-Ser-Gly-Lys(ε-AEEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid);

Compound 17: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Gly-Gly-Lys(ε-AEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid);

Compound 18: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Gly-Gly-Lys(ε-AEEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid);

Compound 19: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Lys(ε-AEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid); and

Compound 20: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Lys(ε-AEEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid).

31. A method for diagnosis, alleviation, improvement or treatment of abnormal symptom or disease in mammals, by administering an efficient amount of the bioactive substance-bioactive substance carrier conjugate according to claim 9 into mammal patients including human in need of administration thereof.

32. The method according to claim 31, wherein the bioactive substance-bioactive substance carrier conjugate contains insulinotropic peptide as a bioactive substance, to treat diabetes, reduce blood glucose, or to stimulate insulin secretion.

33. The method according to claim 32, wherein the bioactive substance-bioactive substance carrier conjugate is selected from the group consisting of:

Compound 1: His-D-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Lys-(ε-AEEA-PDSP)-NH_2.4TFA;

Compound 2: His-D-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Lys-(ε-AEEEA-PDSP)-NH_2.4TFA;

Compound 3: His-D-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-(ε-AEEEA-PDSP)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-NH_2.4TFA;

Compound 4: His-D-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys(ε-AEEEA-PDSP)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-NH_2.4TFA;

Compound 5: His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Lys-(ε-AEEA-PDSP)-NH_2.4TFA;

Compound 6: His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-

Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Lys-(ε-AEEEA-PDSP)-NH_2.4TFA;

Compound 7: His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Lys-(ε-AEEA-PDSP)NH_2.5TFA;

Compound 8: His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Lys-(ε-AEEEA-PDSP)-NH_2.5TFA;

Compound 9: His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-(ε-AEEA-PDSP)-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2.5TFA;

Compound 10: His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-(ε-AEEEA-PDSP)-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2.5TFA;

Compound 11: His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Lys-(ε-AEEA-PDSP)NH_2.5TFA;

Compound 12: His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Lys-(ε-AEEEA-PDSP)-NH_2.5TFA;

Compound 13: His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-(ε-AEEA-PDSP)-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2.5TFA; and

Compound 14: His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-(ε-AEEEA-PDSP)-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2.5TFA.

34. The method according to claim 31 wherein the bioactive substance-bioactive substance carrier conjugate contains LHRH (Luteinizing Hormone-Releasing Hormone) as a bioactive substance, to diagnose or treat a sex hormone-related disease and control ovulation period in mammals,.

35. The method according to claim 34, wherein the sex hormone-related disease is prostate cancer, endometriosis or uterus myoma.

36. The method according to claim 34, wherein the bioactive substance-bioactive substance carrier conjugate is selected from the group consisting of:

Compound 15: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Gly-Ser-Gly-Lys(ε-AEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid, pE);

Compound 16: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Gly-Ser-Gly-Lys(ε-AEEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid);

Compound 17: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Gly-Gly-Lys(ε-AEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid);

Compound 18: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Gly-Gly-Lys(ε-AEEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid);

Compound 19: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Lys(ε-AEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid); and

Compound 20: Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-Lys(ε-AEEEA-PDSP)-NH_2.2TFA (wherein Pyr=pyroglutamic acid).

FIG. 1

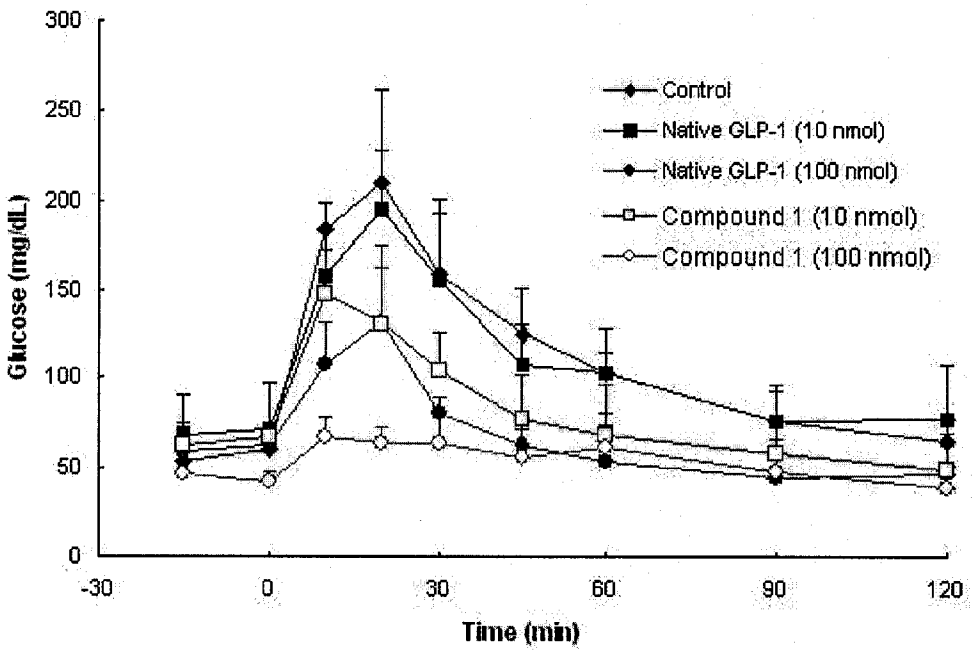

300
250
200
150
100
50
0
Glucose (mg/dL)
-30
0
30
60
90
120
Time (min)
Control
Native GLP-1 (10 nmol)
Native GLP-1 (100 nmol)
Compound 1 (10 nmol)
Compound 1 (100 nmol)

FIG. 2

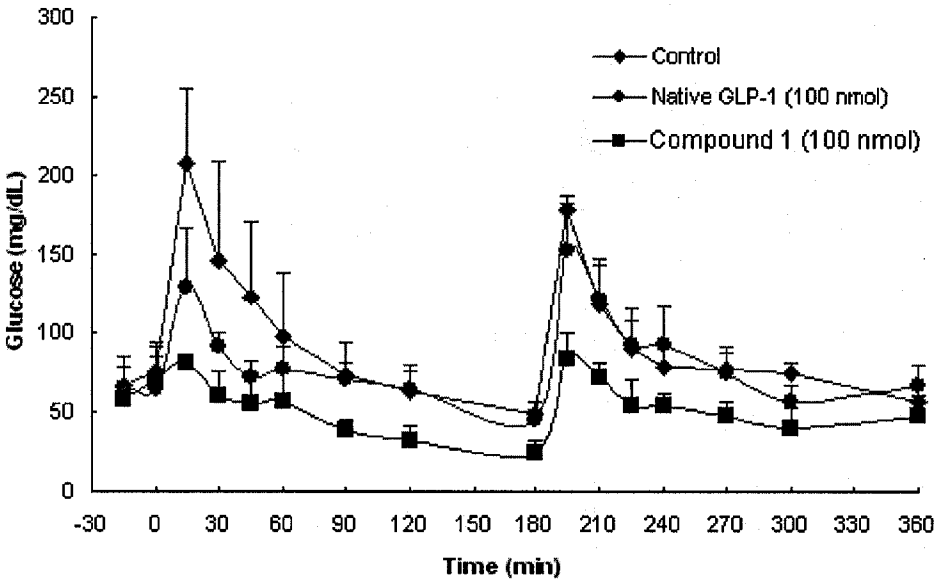

Control
Native GLP-1 (100 nmol)
Compound 1 (100 nmol)
Glucose (mg/dL)
300
250
200
150
100
50
0
-30
0
30
60
90
120
150
180
210
240
270
300
330
360
Time (min)

FIG. 3

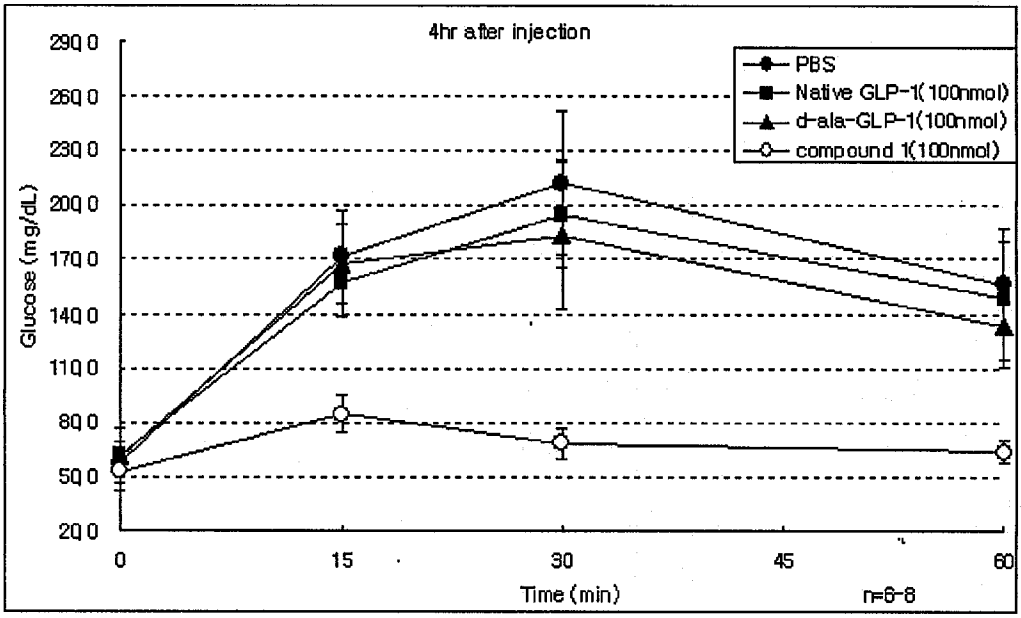
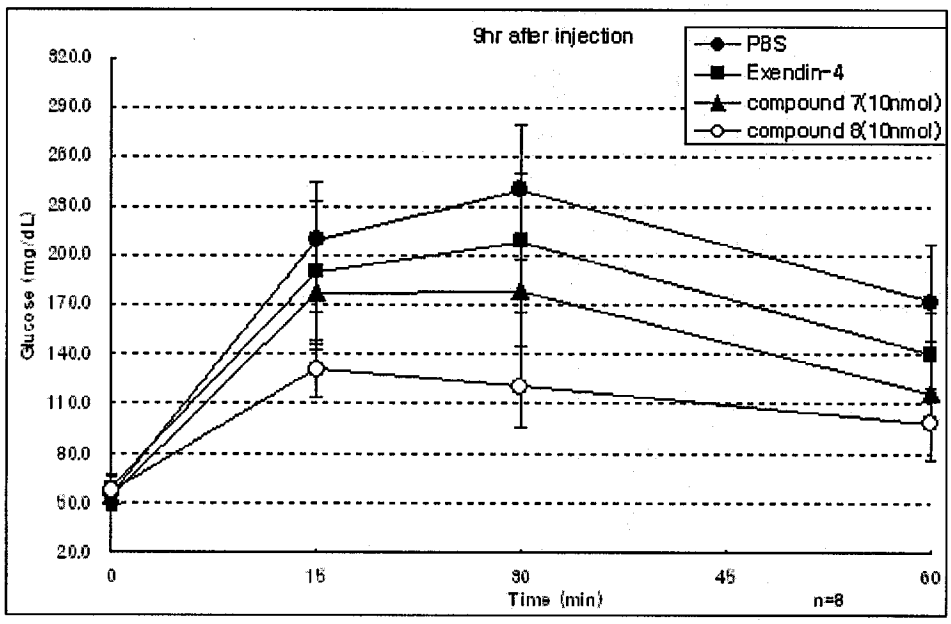

4hr after injection
PBS
Native GLP-1(100nmol)
d-ala-GLP-1(100nmol)
compound 1(100nmol)
Glucose (mg/dL)
290 0
260 0
230 0
200 0
170 0
140 0
110 0
80 0
50 0
20 0
0
15
30
45
60
Time (min)
n=6-8

FIG. 4


9hr after injection
PBS
Exendin-4
compound 7(10nmol)
compound 8(10nmol)
320.0
290.0
260.0
230.0
200.0
170.0
140.0
110.0
80.0
50.0
20.0
Glucose (mg/dL)
0
15
30
45
60
Time (min)
n=8

<110> PEPTRON CO., LTD

<120> BIOACTIVE SUBSTANCE CARRIER FOR IN VIVO STABLE DELIVERY THEREOF, CONJUGATE CONTAINING THE SAME, AND METHOD OF IN VIVO STABLE DELIVERY OF THE BIOACTIVE SUBSTANCE

<130> OPP20061826KR

<150> US 60/731,592
<151> 2005-10-27

<160> 20

<170> KopatentIn 1.71

<210> 1
<211> 31
<212> PRT
<213> Artificial Sequence

<220>
<223> Amino acid sequence of D-Ala8-GLP-1(7-36)-Lys37

<220>
<221> MUTAGEN
<222> (2)
<223> transformed into D-form

<220>
<221> MUTAGEN
<222> (31)
<223> Lys to which a linker and a reactive group represented by (e-AEEA-PDSP) (e: epsilon) are attached.

<400> 1
His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly
1 5 10 15

Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Arg Lys
20 25 30

<210> 2

<211> 31
<212> PRT
<213> Artificial Sequence

<220>
<223> Amino acid sequence of
D-Ala8-GLP-1(7-36)-Lys37-(e-AEEEA-PDSP)-NH2.4TFA (e: epsilon)

<220>
<221> MUTAGEN
<222> (2)
<223> transformed into D-form

<220>
<221> MUTAGEN
<222> (31)
<223> Lys to which a linker and a reactive group represented by
(e-AEEEA-PDSP) (e: epsilon) are attached.

<400> 2
His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly
1 5 10 15

Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Arg Lys
20 25 30

<210> 3
<211> 30
<212> PRT
<213> Artificial Sequence

<220>
<223> Amino sequence of D-Ala8-Lys26-(e-AEEA-PDSP)-GLP-1(7-36)-NH2.4TFA
(e: epsilon)

<220>
<221> MUTAGEN
<222> (2)
<223> transformed into D-form

<220>
<221> MUTAGEN
<222> (20)
<223> Lys to which a linker and a reactive group represented by (e-AEEA-PDSP) (e: epsilon) are attached.

<400> 3
His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly
1 5 10 15

Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Arg
20 25 30

<210> 4
<211> 30
<212> PRT
<213> Artificial Sequence

<220>
<223> Amino acid sequence of D-Ala8-Lys26-(e-AEEEA-PDSP)-GLP-1(7-36)-NH2.4TFA (e: epsilon)

<220>
<221> MUTAGEN
<222> (2)
<223> transformed into D-form

<220>
<221> MUTAGEN
<222> (20)
<223> Lys to which a linker and a reactive group represented by (e-AEEEA-PDSP) (e: epsilon) are attached.

<400> 4
His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly
1 5 10 15

Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Arg
20 25 30

<210> 5
<211> 31

<212> PRT
<213> Artificial Sequence

<220>
<223> Amino acid sequence of GLP-1(7-36)-Lys37-(e-AEEA-PDSP)-NH2.4TFA
(e: epsilon)

<220>
<221> MUTAGEN
<222> (31)
<223> Lys to which a linker and a reactive group represented by
(e-AEEA-PDSP) (e: epsilon) are attached.

<400> 5
His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly
1 5 10 15

Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Arg Lys
20 25 30

<210> 6
<211> 31
<212> PRT
<213> Artificial Sequence

<220>
<223> Amino acid sequence of GLP-1(7-36)-Lys37-(e-AEEEA-PDSP)-NH2.4TFA
(e: epsilon)

<220>
<221> MUTAGEN
<222> (31)
<223> Lys to which a linker and a reactive group represented by
(e-AEEEA-PDSP) (e: epsilon) are attached.

<400> 6
His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly
1 5 10 15

Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Arg Lys
20 25 30

<210> 7
<211> 40
<212> PRT
<213> Artificial Sequence

<220>
<223> Amino acid sequence of Exendin-4
(1-39)-Lys40-(e-AEEA-PDSP)-NH2.5TFA (e: epsilon)

<220>
<221> MUTAGEN
<222> (40)
<223> Lys to which a linker and a reactive group represented by
(e-AEEA-PDSP)-NH2.4TFA (e: epsilon) are attached.

<400> 7
His Gly Glu Gly Thr Phe Thr Ser Asp Leu Ser Lys Gln Met Glu Glu
1 5 10 15

Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser
20 25 30

Ser Gly Ala Pro Pro Pro Ser Lys
35 40

<210> 8
<211> 40
<212> PRT
<213> Artificial Sequence

<220>
<223> Amino acid sequence of
Exendin-4(1-39)-Lys40-(e-AEEEA-PDSP)-NH2.5TFA (e: epsilon)

<220>
<221> MUTAGEN
<222> (40)
<223> Lys to which a linker and a reactive group represented by
(e-AEEEA-PDSP)-NH2.5TFA (e: epsilon) are attached.

<400> 8
His Gly Glu Gly Thr Phe Thr Ser Asp Leu Ser Lys Gln Met Glu Glu
1 5 10 15

Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser
20 25 30

Ser Gly Ala Pro Pro Pro Ser Lys
35 40

<210> 9
<211> 39
<212> PRT
<213> Artificial Sequence

<220>
<223> Amino acid sequence of
Lys27-(e-AEEA-PDSP)-Exendin-4(1-39)-NH2.5TFA (e: epsilon)

<220>
<221> MUTAGEN
<222> (27)
<223> Lys to which a linker and a reactive group represented by
(e-AEEA-PDSP) (e: epsilon) are attached.

<400> 9
His Gly Glu Gly Thr Phe Thr Ser Asp Leu Ser Lys Gln Met Glu Glu
1 5 10 15

Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser
20 25 30

Ser Gly Ala Pro Pro Pro Ser
35

<210> 10
<211> 39
<212> PRT
<213> Artificial Sequence

<220>
<223> Amino acid sequence of Lys27-(e-AEEEA-PDSP)-Exendin-4
(1-39)-NH2.5TFA (e: epsilon)

<220>
<221> MUTAGEN
<222> (27)
<223> Lys to which a linker and a reactive group represented by (e-AEEEA-PDSP) (e: epsilon) are attached.

<400> 10
His Gly Glu Gly Thr Phe Thr Ser Asp Leu Ser Lys Gln Met Glu Glu
1 5 10 15

Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser
20 25 30

Ser Gly Ala Pro Pro Pro Ser
35

<210> 11
<211> 40
<212> PRT
<213> Artificial Sequence

<220>
<223> Amino acid sequence of Exendin-3(1-39)-Lys40-(e-AEEA-PDSP)-NH2.5TFA (e: epsilon)

<220>
<221> MUTAGEN
<222> (40)
<223> Lys to which a linker and a reactive group represented by (e-AEEA-PDSP) (e: epsilon) are attached.

<400> 11
His Ser Asp Gly Thr Phe Thr Ser Asp Leu Ser Lys Gln Met Glu Glu
1 5 10 15

Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser
20 25 30

Ser Gly Ala Pro Pro Pro Ser Lys
35 40

<210> 12
<211> 40
<212> PRT
<213> Artificial Sequence

<220>
<223> Amino acid sequence of Exendin-3 (1-39)-Lys40-(e-AEEEA-PDSP)-NH2.5TFA (e: epsilon)

<220>
<221> MUTAGEN
<222> (40)
<223> Lys to which a linker and a reactive group represented by (e-AEEEA-PDSP) (e: epsilon) are attached.

<400> 12
His Ser Asp Gly Thr Phe Thr Ser Asp Leu Ser Lys Gln Met Glu Glu
1 5 10 15

Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser
20 25 30

Ser Gly Ala Pro Pro Pro Ser Lys
35 40

<210> 13
<211> 39
<212> PRT
<213> Artificial Sequence

<220>
<223> Amino acid sequence of Lys27-(e-AEEA-PDSP)-Exendin-3 (1-39)-NH2.5TFA (e: epsilon)

<220>
<221> MUTAGEN
<222> (27)
<223> Lys to which a linker and a reactive group represented by (e-AEEA-PDSP) (e: epsilon) are attached.

<400> 13
His Ser Asp Gly Thr Phe Thr Ser Asp Leu Ser Lys Gln Met Glu Glu
1 5 10 15

Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser
20 25 30

Ser Gly Ala Pro Pro Pro Ser
35

<210> 14
<211> 39
<212> PRT
<213> Artificial Sequence

<220>
<223> Amino acid sequence of
Lys27-(e-AEEEA-PDSP)-Exendin-3(1-39)-NH2.5TFA (e: epsilon)

<220>
<221> MUTAGEN
<222> (27)
<223> Lys to which a linker and a reactive group represented by
(e-AEEEA-PDSP) (e: epsilon) are attached.

<400> 14
His Ser Asp Gly Thr Phe Thr Ser Asp Leu Ser Lys Gln Met Glu Glu
1 5 10 15

Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser
20 25 30

Ser Gly Ala Pro Pro Pro Ser
35

<210> 15
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Amino acid sequence of Leuprolide-GSG-Lys-(e-AEEA-PDSP)-NH2.2TFA
(e: epsilon)

<220>
<221> MUTAGEN

<222> (1)
<223> Modified Glu (pyroglutamic acid)

<220>
<221> MUTAGEN
<222> (6)
<223> transformed into D-form

<220>
<221> MUTAGEN
<222> (13)
<223> Lys to which a linker and a reactive group represented by (e-AEEA-PDSP) (e: epsilon) are attached.

<400> 15
Glu His Trp Ser Tyr Leu Leu Arg Pro Gly Ser Gly Lys
1 5 10

<210> 16
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Amino acid sequence of Leuprolide-GSG-Lys-(e-AEEEA-PDSP)-NH2.2TFA (e: epsilon)

<220>
<221> MUTAGEN
<222> (6)
<223> transformed into D-form

<220>
<221> MUTAGEN
<222> (13)
<223> Lys to which a linker and a reactive group represented by (e-AEEEA-PDSP) (e: epsilon) are attached.

<400> 16
Glu His Trp Ser Tyr Leu Leu Arg Pro Gly Ser Gly Lys
1 5 10

<210> 17
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
<223> Amino acid sequence of Leuprolide-GG-Lys-(e-AEEA-PDSP)-NH2.2TFA (e: epsilon)

<220>
<221> MUTAGEN
<222> (1)
<223> Modified Glu (pyroglutamic acid)

<220>
<221> MUTAGEN
<222> (6)
<223> transformed into D-form

<220>
<221> MUTAGEN
<222> (12)
<223> Lys to which a linker and a reactive group represented by (e-AEEA-PDSP) (e: epsilon) are attached.

<400> 17
Glu His Trp Ser Tyr Leu Leu Arg Pro Gly Gly Lys
1 5 10

<210> 18
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
<223> Amino acid sequence of Leuprolide-GG-Lys-(e-AEEEA-PDSP)-NH2.2TFA (e: epsilon)

<220>
<221> MUTAGEN

<222> (1)
<223> Modified Glu (pyroglutamic acid)

<220>
<221> MUTAGEN
<222> (6)
<223> Transformed into D-form

<220>
<221> MUTAGEN
<222> (12)
<223> Lys to which a linker and a reactive group represented by (e-AEEEA-PDSP) (e: epsilon) are attached.

<400> 18
Glu His Trp Ser Tyr Leu Leu Arg Pro Gly Gly Lys
1 5 10

<210> 19
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Amino acid sequence of Leuprolide-Lys-(e-AEEA-PDSP)-NH2.2TFA (e: epsilon)

<220>
<221> MUTAGEN
<222> (1)
<223> Modified Glu (pyroglutamic acid)

<220>
<221> MUTAGEN
<222> (6)
<223> transformed into D-form

<220>
<221> MUTAGEN
<222> (10)
<223> Lys to which a linker and a reactive group represented by

(e-AEEA-PDSP) (e: epsilon) are attached.

<400> 19
Glu His Trp Ser Tyr Leu Leu Arg Pro Lys
1 5 10

<210> 20
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Amino acid sequence of Leuprolide-Lys-(e-AEEEA-PDSP)-NH2.2TFA (e: epsilon)

<220>
<221> MUTAGEN
<222> (1)
<223> Modified Glu (pyroglutamic acid)

<220>
<221> MUTAGEN
<222> (6)
<223> transformed into D-form

<220>
<221> MUTAGEN
<222> (10)
<223> Lys to which a linker and a reactive group represented by (e-AEEEA-PDSP) (e: epsilon) are attached.

<400> 20
Glu His Trp Ser Tyr Leu Leu Arg Pro Lys
1 5 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2006/004428

A. CLASSIFICATION OF SUBJECT MATTER

A61K 38/38(2006.01)i, A61K 38/16(2006.01)i, A61P 3/10(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC8 as above

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean Patents and applications for inventions since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search terms used)
eKIPASS, Pubmed (polypeptide, conjugate, linker, blood component, half-life, stability)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00/69900 A2 (CONJUCHEM, INC.) 23 November 2000 See abstract, page 20, line 26 - page 21, line 3, and page 26, line 3- page 27, line 7.	1 - 36
X	WO 04/081053 A1 (HANMI PHARM. CO. LTD.) 23 September 2004 See abstract and claim 16.	1 - 36
A	WO 03/006501 A2 (MAXYGEN HOLDINGS. LTD.) 23 January 2003 See the whole document.	1 - 36
A	WO 01/92584 A1 (EIDGENOSSISCHE TECHNISCHE HOCHSCHULE ZURICH, UNIVERSITAT ZURICH) 6 December 2001 See the whole document.	1 - 36

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 DECEMBER 2006 (18.12.2006)	**18 DECEMBER 2006 (18.12.2006)**
Name and mailing address of the ISA/KR Korean Intellectual Property Office 920 Dunsan-dong, Seo-gu, Daejeon 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Authorized officer LEE, Mi Jeong Telephone No. 82-42-481-5601

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2006/004428

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of :

   a. type of material
   - [x] a sequence listing
   - [ ] table(s) related to the sequence listing

   b. format of material
   - [ ] on paper
   - [x] in electronic form

   c. time of filing/furnishing
   - [ ] contained in the international application as filed
   - [x] filed together with the international application in electronic form
   - [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2006/004428

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 13-21, 31-36
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 13-21, 31-36 are directed to methods for treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR2006/004428

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 00/69900 A2	23.11.2000	US 20060135428 A1	22.06.2006
		US 6849714 B1	01.02.2005
		JP 2005255689 A2	22.09.2005
		EP 1180121 A1	20.02.2002
		EP 1105409 A2	13.06.2001
		CN 1350548 A	22.05.2002
		AU 0051393 A5	05.12.2000
WO 04/081053 A1	23.09.2004	US 20040180054 A1	16.09.2004
		EP 1601698 A1	07.12.2005
		CN 1761684 A	19.04.2006
		AU 4220163 AA	23.09.2004
WO 03/006501 A2	23.01.2003	US 20020004483 A1	10.01.2002
		JP 2003519478 T2	24.06.2003
		EP 1425304 A2	09.06.2004
		CN 1404401 T	19.03.2003
		AU 0123533 A5	24.07.2001
WO 01/92584 A1	06.12.2001	US 20030220245 A1	27.11.2003
		JP 2003535066 T2	25.11.2003
		EP 1292709 A1	19.03.2003
		AU 0175226 A5	11.12.2001